

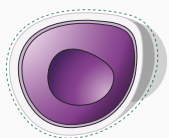
Scientific Report 2009-2010



Welcome to the CNB 2



1 Immunology & Oncology 4



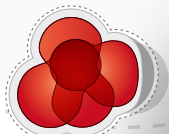
2 Cellular & Molecular Biology 44



3 Microbial Biotechnology 76



4 Plant Molecular Genetics 102



5 Macromolecular Structures 126



6 Systems Biology 152



7 Emerging Scientists 160



8 Research Support 166



9 Annexes 184

Welcome
to the CNB

CNB DIRECTOR

Jose María Valpuesta Moralejo

The CNB during 2009-2010

Although our institution, the Spanish National Research Council (CSIC), is going through a difficult period due to a decrease in the budget (around 15% last year), the Centro Nacional de Biotecnología (CNB) is enduring the economic crisis with a certain ease, thanks to the energy and dynamism of a scientific staff of 68 group leaders.

Over the last two years, this multidisciplinary group of people (working in the fields of Structural Biology, Cell Biology, Virology, Microbiology, Plant Biology, Immunology, Oncology and Systems Biology) have contributed to the publication of almost 400 ISI papers, have obtained 120 grants (20 from the EU VII framework program), have submitted 58 PhD theses and have taught more than 400 hours in master's degree programs and workshops in Spain and abroad.

This has served, among other things, to help in the training of 120 predoctoral students, 30 of which are funded by the "La Caixa" international fellowship programme. Likewise, the outreach effort of our scientists persists; the CNB has hosted 270 seminars and its scientists have organised 32

workshops in the centre. All this effort would not have been possible without the support of the excellent facilities at the CNB and the efficiency of the administrative staff.

As a biotechnological centre, there is a certain urge to translate the knowledge generated into something useful to society. In this line, during the past two years CNB scientists have applied for 12 patents (10 international), of which one has already been licensed, and have generated 58 contracts with private and public institutions.

This effort has not passed unnoticed to our Scientific Advisory Board, which last year subjected the centre, its departments and its scientists to a five-year evaluation. The results were in general excellent, although the SAB also detected problems associated with the persistent bureaucratic rigidity of the Spanish scientific system, and the shortage in the infrastructure and personnel provided by the CSIC. This latter problem could be at least partially solved by the establishment on the campus of the Universidad Autónoma de Madrid (UAM), where the CNB is located, of new technological platforms that should help strengthen the links between the UAM and the CSIC, and reinforce the ambitious plan of making the "UAM+CSIC International Campus of Excellence" one of the most important ones in the field of biology in Europe.



**Inmunology
Oncology**

The Department of Immunology and Oncology (DIO) was created in 1996, with three main objectives:

- To contribute to the generation of scientific knowledge
- To improve society's wellbeing through biomedical research
- To collaborate with Spanish biotech companies to increase their competitiveness

Since its inception, the DIO has combined academic research and the establishment of strategic alliances with pharmaceutical and technology-based companies to seek synergies and to respond to the translational vocation of its scientists. The goal of DIO scientists is to develop new agents for the treatment of some of the major diseases that affect humankind in the 21st century, such as chronic inflammatory, infectious and autoimmune diseases, as well as cancer.

In this report, we summarize the 19 research projects under way in the department. In many of these projects, DIO researchers conjugate molecular analysis of the intracellular signal transduction pathways that control cell migration, differentiation, survival, proliferation and death, with the development of animal models that resemble human disease. The DIO has extensive experience with mouse models of systemic lupus erythematosus, rheumatoid arthritis, asthma, inflammatory bowel disease, multiple sclerosis, type I diabetes, and a number of models for cancer (breast, colon, leukaemia, etc.).

Last, but not less important, is our commitment to the preparation of new generations of scientists. Many DIO members participate in teaching activities, lectures and seminars in national and international institutions. Fourteen PhD dissertations directed by DIO scientists were defended in 2009-2010; most of these new young scientists are now postdoctoral researchers in international institutions, spreading the DIO values beyond our doors. We are indebted to all of them as well as to the technical, editorial and administrative staff, whose contribution makes possible the quality of work developed at the DIO.

CARLOS ARDAVÍN	Diferentiation and Functional Specialization of Dendritic Cells during Inflammatory, Infectious and Allergic Processes	6
DIMITRI BALOMENOS	Interplay of Activation, Apoptosis and Cell Regulators Monitors Autoimmune T Cell Memory Responses and Inflammation	8
DOMINGO F. BARBER CASTAÑO	Lymphocytes in Physiological and Pathological Processes: Autoimmune Inflammatory Diseases, Cancer Immunotherapy, and Nanomedicine	10
YOLANDA R. CARRASCO	B Cell Dynamics	12
ANA CLARA CARRERA	PI3K in Cancer and Inflammation	14
ANA CUENDA	Stress-Activated Protein Kinase: p38MAPK Signalling Pathways and their Role in Human Diseases	16
THIERRY J. FISCHER	Chemokine-Mediated Cell Migration and Endocytosis	18
ANA GONZÁLEZ	The Role of Ras Effectors in Inflammation and Cancer	20
LEONOR KREMER	Chemokine Receptors in Cancer Biology	22
SANTOS MAÑES BROTON	Signalling Networks in Inflammation and Cancer	24
CARLOS MARTÍNEZ ALONSO & KAREL H.M. VAN WELY	Linking Centrosomal Aberrations to Chromosomal Instability	26
MARIO MELLADO	Chemokine Receptors: New Targets for Therapeutic Intervention	28
ISABEL MÉRIDA	Lipid Signalling	30
IGNACIO MORENO DE ALBORÁN	Function of the c-Myc Proto-Oncogene <i>in vivo</i>	32
LOURDES PLANELLES	Function and Regulation of APRIL, a TNF Protein: Implications in Pathology	34
HUGH REYBURN	Receptor-Ligand Interactions in Immune Responses to Cancer and Viruses	36
JOSÉ MIGUEL RODRÍGUEZ FRADE	Chemokine Signalling	38
JESÚS MARÍA SALVADOR	T cell Signalling in Autoimmune Diseases and Cancer	40
MAR VALÉS GÓMEZ	Biochemical Characterization of the Ligands for the Immune Receptor NKG2D: Implications of their Heterogeneity for Pathology and Therapy	42

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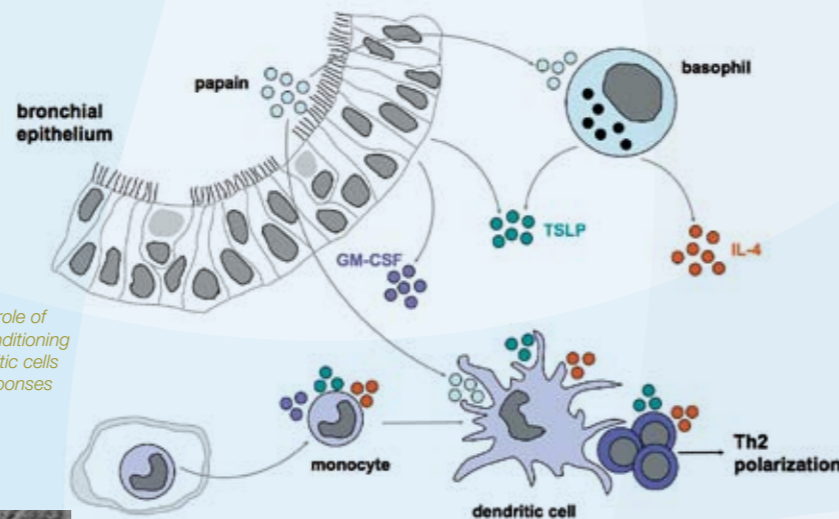
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Differentiation and Functional Specialization of Dendritic Cells during Inflammatory, Infectious and Allergic Processes

Our research aims at exploring the functional specialization of dendritic cells derived from monocytes during inflammatory responses caused by bacterial, yeast and parasitic infections, or by allergic reactions induced by allergens derived from plants, fungi or acaridae.



Working hypothesis on the role of the cytokine IL-4 on the conditioning of monocyte-derived dendritic cells for the induction of Th2 responses against allergens.

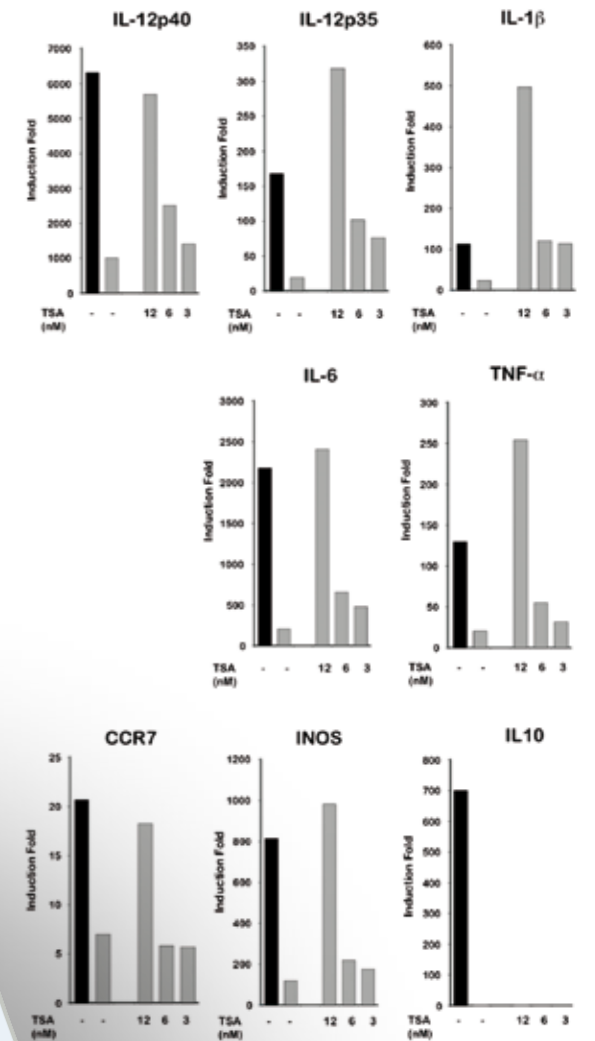
In particular our current research interests are focused on the following topics, that reflect the developmental and functional plasticity of monocytes and the relevance of monocyte-derived dendritic cells during infectious and allergic processes:

- Analysis of the effector functions of mouse monocytes in innate and adaptive immunity.
- Analysis of the differential migratory properties of mouse monocyte-derived dendritic cells and macrophages.
- Regulation of mouse monocyte differentiation into dendritic cells and macrophages during in vivo immune response to *Leishmania major*.

- Functional specialization of mouse dendritic cells for the induction of Th2 responses against pathogens and allergens.
- Gene expression profile of mouse monocytes and monocyte-derived dendritic cells exposed to allergens and Th2-polarizing mediators.
- Effect of statin treatment on proinflammatory cytokine production and nitric oxide metabolism by LPS-activated or *Listeria monocytogenes*-infected mouse monocyte-derived DCs.
- Analysis of the splenic innate immune response to in vivo *Listeria monocytogenes* infection on statin-treated mice.
- Role of type-I interferon in the induction of Th17 immune responses against *Candida albicans* in mice.

The methodology designed for addressing these objectives involves the following experimental approaches:

- Development of in vitro and in vivo infection models in different mouse strains, including mice deficient in cytokines (GM-CSF), cytokine/chemokine receptors (IFNAR, CCR2, CCR7), molecules involved in dendritic cell activation (MyD88, NOD 1/2, IRF-3, PPAR-γ) and mice transgenic for TCRs specific for ovalbumin-derived peptides expressed on MHC I (OT-I) or MHC II (OT-II, DO.11).
- Cell biology techniques designed for the purification or isolation of defined cell populations from mouse bone marrow, skin, lymph nodes and spleen, involving magnetic bead and FACS cell separation methods.
- Analysis of monocytes, dendritic cells and macrophages from *Leishmania*- or *Listeria*-infected mice on cell suspensions or tissue sections, by electron microscopy or confocal microscopy after immunofluorescent staining.
- In vitro differentiation of dendritic cells and macrophages on GM-CSF, IL-3 or Flt3L-driven cultures from monocytes or bone marrow precursors.
- Analysis of gene expression profiles at the protein level by flow cytometry, ELISA and electrophoresis.
- Analysis of gene expression at the mRNA level by real-time quantitative PCR, whole mouse genome microarray analyses and chromatin immunoprecipitation.



Epigenetic regulation of Th2 responses: reversal by the histone deacetylase inhibitor TSA of IL-4-dependent inhibition of pro-inflammatory cytokine production induced by TLR-4 engagement in monocyte-derived dendritic cells.

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Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. Domínguez MP, Ardavín C. **Immunological Reviews** 234:90-104 (2010).



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Interplay of Activation, Apoptosis and Cell Regulators Monitors Autoimmune T Cell Memory Responses and Inflammation

T cell memory responses and Homeostasis in Immunity and Autoimmunity.

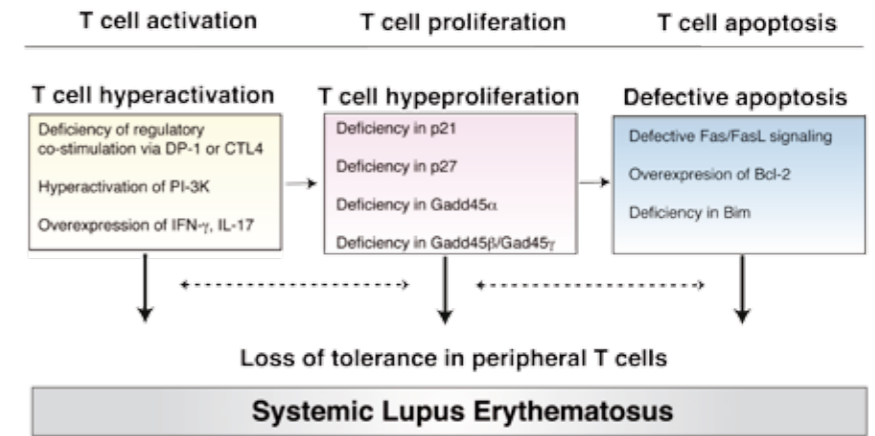
Apoptosis is considered a basic mechanism for limiting T cell memory expansion known as homeostasis. Nevertheless, we find that the control of activation of memory T cells and of proliferation are also fundamental for memory T cell homeostasis (Fig. 1). We suggest that memory responses and homeostasis require a combination of control in activation, apoptosis and proliferation (3).

The cell cycle inhibitor p21 suppresses autoimmunity and controls T cell memory responses.

We have shown that p21 is an autoimmunity suppressor, since p21 deficiency leads to autoimmunity (Balomenos D et al. Nat Med 2000, Arias CF et al. J Immunol. 2007. 178:2296-306). Indeed, p21 overexpression in T cells of autoimmune lupus-prone Fas-deficient (lpr/lpr) mice reduces autoimmunity development. (5) by limiting the expansion of autoreactive lpr/lpr memory T cells. p21 plays an attenuating role in T cell expansion upon persistent stimulation of T cells through TCR. Our recent work unveils a novel function of p21 regulating of TCR-dependent activation of memory T cells. Thus we propose that p21 exerts two functions in the response of memory T cells, by first regulating their level of activation and second by controlling their proliferative potential. We are now studying the mechanism by which p21 controls memory T cell activation.

An alternative function for the Fas/FasL apoptosis system. Our analysis of memory T cell activation, apoptosis and cell cycle regulation events in immunity and autoimmune disease, have revealed a previously unknown role for Fas. Similarly, to the function of p21 as a regulator of repeatedly

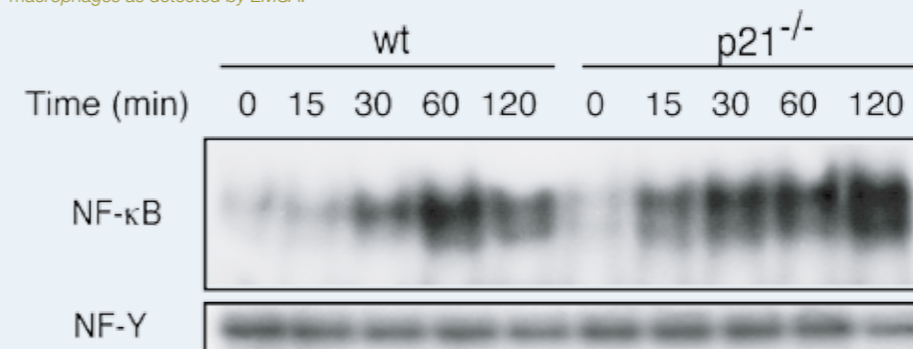
A schematic representation showing that defects in the control of T cell activation, apoptosis and proliferation may lead to Lupus autoimmunity.



activated T cells we have established that Fas also plays a crucial attenuating role in the response of previously activated T cells but not of primary T cells. We are currently investigating the mechanistic aspects of this new role of the Fas/FasL system.

p21 regulates the macrophage activation pathway. p21 has a general role in the immune response since independently of its cell cycle inhibitory capacity, regulates macrophage activation by controlling the (NF- κ B) pathway in the cytoplasm (1, Fig. 2). p21 regulation of NF- κ B activation is critical for progression of in vivo inflammation, since it decreases sensitivity to LPS-induced septic shock. We are studying the mechanism that defines the role of p21 in NF- κ B activation, and whether p21 suppresses glomerulonephritis development through its regulatory effect on innate immunity inflammation.

Lack of p21 leads to an increased activation of the NF- κ B pathway in LPS-stimulated macrophages as detected by EMSA.



Inmunology Oncology

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Lymphocytes in Physiological and Pathological Processes: Autoimmune Inflammatory Diseases, Cancer Immunotherapy, and Nanomedicine

Molecular and cellular mechanisms in autoimmune disease: identifying strategies for therapeutic intervention.

Approximately 5-10% of the population in the developed world is affected by at least one of >80 autoimmune disorders; these chronic, debilitating diseases have an enormous social and economic impact. To analyse the mechanisms that operate in autoimmune diseases and identify new drug targets and therapeutic strategies, we study intracellular signaling pathways that induce autoimmunity or inflammation when hyperactivated, as well as the mechanisms that maintain peripheral tolerance in the immune system.

Our lab studies several aspects of the initiation and progression of autoimmune disease in murine models: 1) the role of p110 γ PI3K in T cell activation and autoimmunity, 2) the role of p110 γ PI3K in secondary lymphoid organs and in the onset/ progression of immune responses and autoimmunity, 3) how p85 γ PI3K contributes to CD28 costimulation in the activation of effector and regulatory T cells, and how its absence affects rheumatoid arthritis onset and development, and 4) crosstalk between negative regulators of T cell activation that enforce T cell quiescence and PI3K isoforms, and how crosstalk affects autoimmunity.

NKG2D in autoimmunity and tumour immunotherapy

NKG2D is an activating receptor expressed by natural killer (NK) cells and T cells; it is implicated in immune responses to infections and tumours and in autoimmunity. NKG2D ligands are not expressed by most normal cells but are up-regulated on numerous tumor cell. In addition, their inappropriate expression in certain tissues can trigger or

exacerbate autoimmune disease. In fact, implicating NKG2D and its ligands in the pathogenesis of several autoimmune diseases.

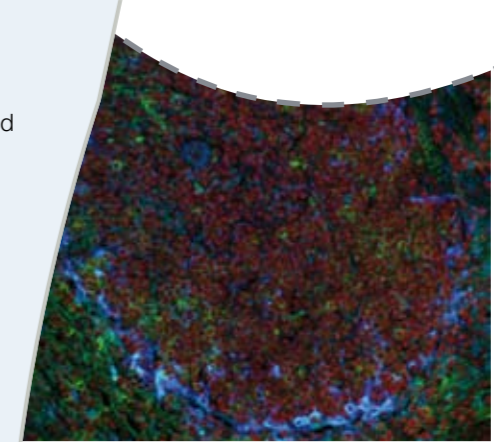
Our projects study the role of NKG2D in autoimmunity and tumour immunotherapy: 1) NKG2D associates with the adaptor protein DAP10, which binds and activates the PI3K p85 subunit. We are analyzing the specific contribution of each PI3K isoform to cell activation via NKG2D. 2) To study the contribution of NKG2D to the initiation/progression of autoimmune processes, we analyse NKG2D ligand expression in several mouse models of autoimmune disease, and study the correlation between ligand expression and disease onset/severity.

Nanomedicine as a therapeutic approach for treating cancer and autoimmune disease

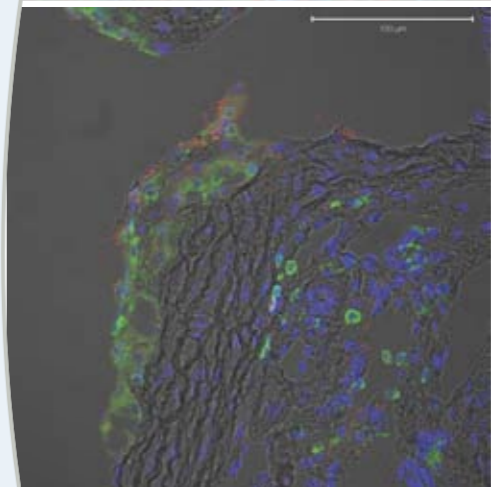
Radiotherapy- and chemotherapy-based cancer treatments affect both tumours and healthy tissue, leading to a search for more specific ways to fight tumours. Tumour immunotherapy is a promising treatment strategy, as it could enhance the immune system's natural capacity to control tumour development. Studies suggest IFN- γ as effective in tumour elimination, although it is difficult to deliver an appropriate cytokine dose to the tumour without causing toxicity to surrounding tissues. Directed targeting to the tumour could improve the efficiency of its delivery, increasing local dosage without augmenting the systemic concentration. Nanotechnology provides a means to target drugs using superparamagnetic iron oxide nanoparticles as drug delivery systems in conjunction with a magnetic field, applied externally or implanted internally.

In mouse models of cancer, we tested uniform dimercaptosuccinic acid (DMSA)-coated monodisperse magnetic nanoparticles (MNP) as a delivery system for IFN- γ . IFN- γ -adsorbed DMSA-coated MNP were targeted to the tumour site by applying an external magnetic field. We found nanoparticle accumulation and cytokine delivery at the tumour site, which led to increased T cell and macrophage infiltration and promoted an antiangiogenic effect, resulting in a notable reduction in tumour size. Our findings show that these nanoparticles can be an efficient in vivo drug delivery system for tumour immunotherapy.

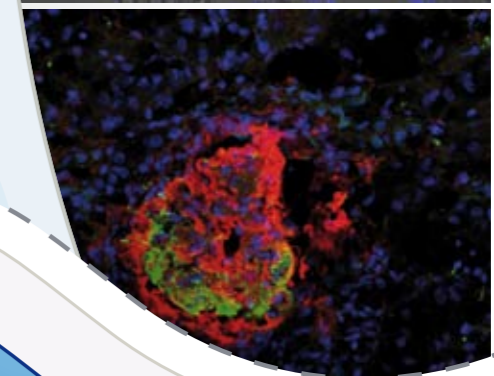
We are also developing and validating a nanoparticle-based system for controlled, localized release of small interfering RNA (siRNA), microRNA, antagomirs and aptamers for specific gene silencing and cell targeting, for treatment of cancer and autoimmunity.



This immunofluorescence staining was performed on spleen frozen sections from p110 $\delta^{WT/WT}$ mice. It shows a germinal center in which p110 δ PI3K is stained in green, marginal metallophilic macrophages in blue and nuclei in red.



This immunofluorescence staining was performed on aortic sections from LDLR^{-/-} p110 γ ^{-/-} mouse embedded in paraffin. A fragment of an atherosclerotic plaque is shown, as well as part of the tunica media and adventitia. T cells are stained in green, nuclei in blue (DAPI) and nuclear proliferation marker Ki67 as well as some erythrocytes in red.



Staining a glomerulus (in red) of a lupus prone MRL/lpr mouse showing expression and co-localization of ult-1 (in green), a Natural Killer cell ligand.

SELECTED PUBLICATIONS

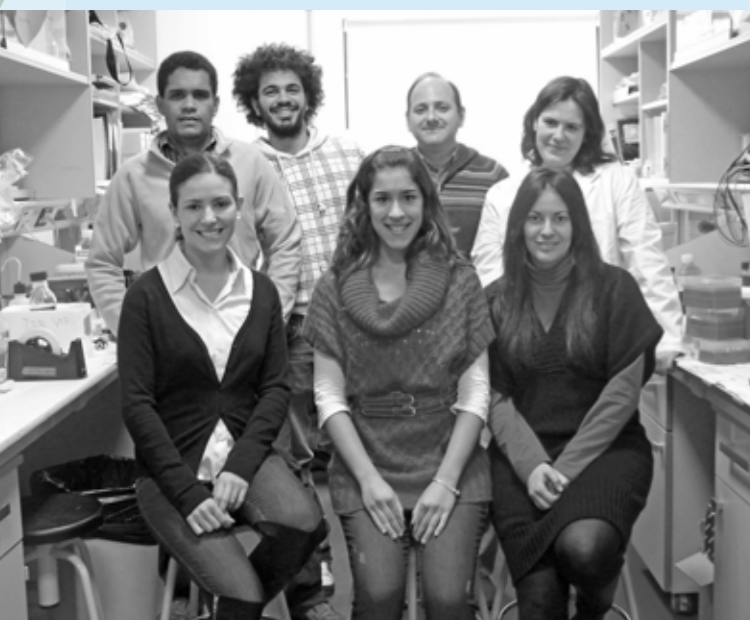
Mejías, R., Pérez-Yagüe, S., Gutiérrez, L., Cabrera, L.I., Spada, R., Acedo, P., Serna, C.J., Lázaro, F.J., Villanueva, A., Morales, M.P., and Barber, D.F. (2011) "Dimercaptosuccinic acid-coated magnetite nanoparticles for magnetically guided in vivo delivery of interferon-gamma for cancer therapy". *Biomaterials* 32, In press, doi:10.1016/j.biomaterials.2011.01.008.

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Alcázar, I., Cortés, I., Zaballos, A., Hernández, C., Fruman, D., Barber, D.F., and Carrera, A.C. (2009) "p85beta phosphoinositide 3-kinase regulates CD28 coreceptor function". *Blood* 113:3198-3208. *Domingo F. Barber and Ana C. Carrera are senior authors on this paper.

PATENT

Morales Herrero, M^a del Puerto; Gómez Roca, Alejandro; Serna Pereda, Carlos; Veintemillas Verdagué, Sabino; Mejías Laguna, Raquel; Pérez Yagüe, Sonia; y Barber Castaño, Domingo F. Magnetic Nanoparticles to be used in a pharmaceutical composition. **P201030138**, filed in Spain. Priority Date: 2th February 2010. Consejo Superior de Investigaciones Científicas. Other countries where the patent has been extended to: Endering in PCT, February 2th 2011.



**Inmunology
Oncology**

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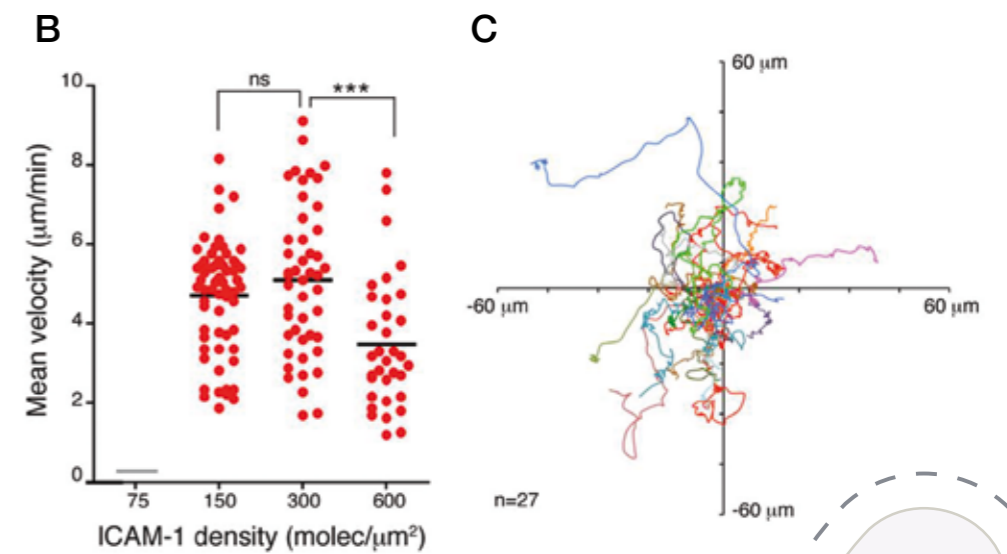
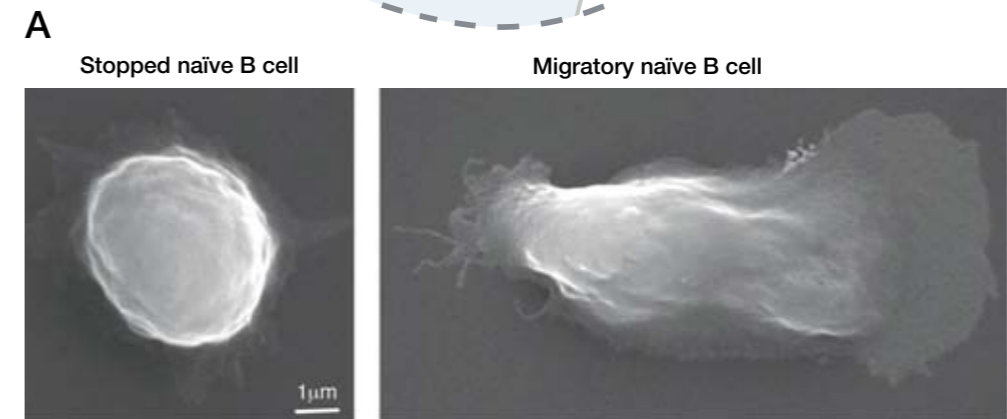
B Cell Dynamics

B cells are essential effectors of the adaptive immune response to pathogens.

They are responsible for pathogen neutralization and clearance through the production of antigen-specific antibodies. The prompt onset of the humoral immune response is thus crucial in the fight against invaders. This process depends critically on the ability of naïve B cells to search for antigen in secondary lymphoid organs (SLO).

Naïve B cells migrate incessantly seeking for specific antigen in SLO. Once they enter lymph nodes through the high endothelial venules, B cells move towards the follicles, guided by the chemokine CXCL13 and a network of stromal cells. B cells explore the entire follicular volume, moving by random walking at an average speed of 6 $\mu\text{m}/\text{min}$. CXCL13, produced mainly by follicular dendritic cells, underlies this B cell behavior by signaling through its receptor, CXCR5. Specific antigen recognition through the B cell receptor (BCR) alters steady-state B cell dynamics at the follicle. B cells stop to gather antigen into a central cluster at the site of contact with the antigen-presenting cell, establishing an immune synapse. Modulation of B cell dynamics thus becomes critical for shaping the process of antigen encounter and subsequent B cell activation.

To dissect the interplay between the BCR and CXCR5 in regulating B cell behavior, we established a two-dimensional model that allows study of CXCL13-mediated B cell migration and antigen encounter in real time (Figure 1). Our results identify a costimulatory function for CXCL13/CXCR5 signaling in BCR-triggered B cell activation by shaping cell dynamics. At limiting conditions of antigen density, naïve B cells establish an LFA-1-supported kinapse with the target membrane in response to CXCL13; through this migratory junction, they encounter antigen and integrate BCR signals. When antigen density is sufficient to trigger a stop signal through the BCR, naïve B cells establish a synapse with the target



*Two-dimensional model to study CXCL13-mediated migration and antigen encounter on naïve B cells. (A) Scanning Electron Microscopy images of two representative naïve B cells, in different dynamic stages (stopped vs. migratory), settled on ICAM-1-containing membranes coated with CXCL13. (B) Values for mean velocity and (C) tracks of migratory B cells on CXCL13-coated ICAM-1-containing membranes. Each dot in (B) corresponds to a single cell; ns, not significant; ***, $p < 0.0001$.*

membrane; CXCR5 signaling then promotes membrane ruffling and LFA-1/ICAM-1 contacts that increase antigen gathering near the synapse and thus, BCR signaling. Both mechanisms require a functional actin cytoskeleton and the activity of the motor protein non-muscle myosin-II. Based on our data, we also propose that B cells exploit both types of dynamic stages, kinapses and synapses, to integrate BCR signals; the use of one or the other will be determined mainly by antigen quality and abundance.

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Depoil D, Weber M, Treanor B, Fleire SJ, Carrasco YR, Harwood NE and Batista FD. Early events of B cell activation by antigen. **Science Signaling**, 2009, Vol 2 (63): pt1.



Inmunology
Oncology

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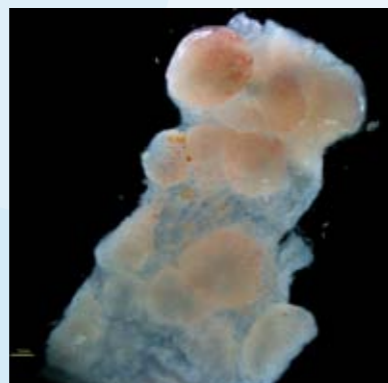
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PI3K in Cancer and Inflammation

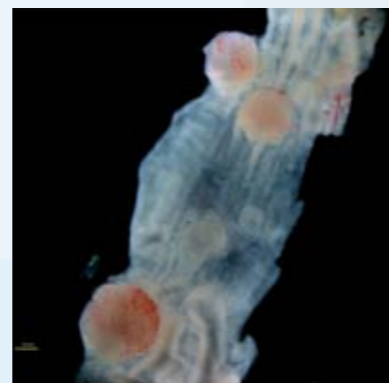
Two biological problems that have occupied the activity of our team (~10 –to-12 members): cancer and inflammation.

Our recent work is based in the assumption that the same biological activities that control physiological responses also control pathology when deregulated. The team is currently working in class I phosphoinositide 3-quinasa (PI3K) with special emphasis in the examining the specific function of each of the four class I PI3K isoforms in physiology and disease.

Reduced colon cancer upon interference with PI3K activation



Colon Carcinoma control mouse



Colon Carcinoma PI3K reduced

Starting with cancer we have recently showed that:

- (1) SADB kinase (that binds PI3K) controls centrosome duplication (*Nature Cell Biol.* 11, 1081-92, 2009).
- (2) PI3K ubiquitous isoforms PI3KCA and CB have different functions in cell division, PI3KCA regulates cell cycle entry and PI3KCB regulates DNA replication (*Mol Cell Biol.* 28: 2803-14,2008; *PNAS*106,7525-30,2009; *PNAS* 107: 7491-6, 2010).
- (3) Interference with PI3K reduces tumor formation *in vivo* (*Gastroenterology*, 138:1374-83, 2010).

These studies contributed to show that PI3K is a target for systemic lupus erithematosus and for cancer treatment and revealed an unexpected nuclear function for PI3KCB.

In Inflammation we previously showed that:

- (1) Activation of T cells *in vivo* (in the mouse) induced by deregulated PI3K activation triggers a Lupus-like disease similar to human Systemic Lupus Erythematosus (SLE) (*FASEB J.* 14,895-903, 2000).
- (2) Deletion or pharmacological inhibition of PI3KCG in the mouse inhibit Lupus (*J. Immunol*176:589-593,2006, *Nature Medicine*11:933-935, 2005).
- (3) PI3K activation is frequent in human SLE (*preliminary results in Nature Medicine* 11:933-935, 2005).

We recently showed that:

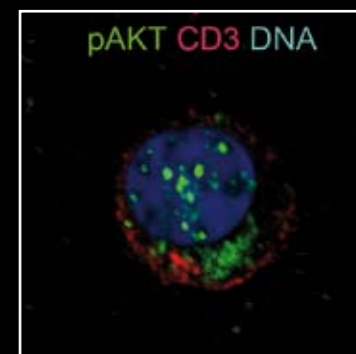
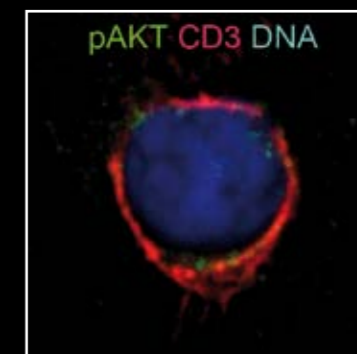
- (4) PI3K involvement in chronic inflammatory disease is at least partially due to its capacity to mediate memory T cell survival (*J. Exp Med* 204:2977-87, 2007; *Blood*,113:3198-208, 2009).

These studies contribute to show that hematopoietic PI3K isoforms are a target for chronic inflammatory disease treatment; we are currently studying human SLE.

Activation of the PI3K/AKT pathway in T cells from Systemic Lupus Erythematosus patients

T cell Control Individul

T cell Systemic Lupus



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PATENT

Patente Biomarcador Cancer 201031137.



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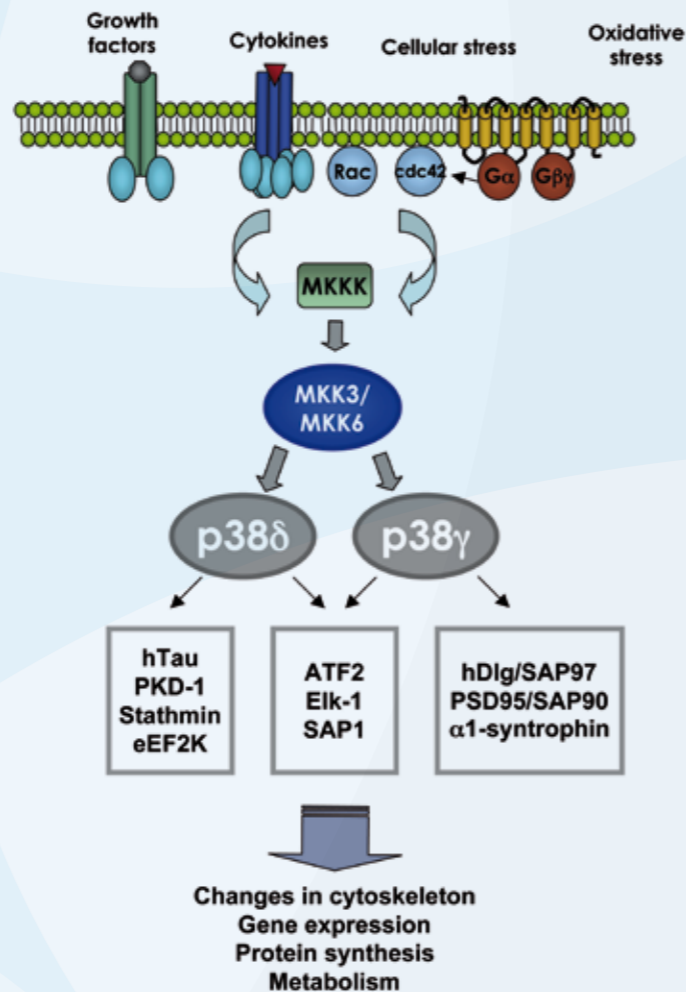
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Stress-Activated Protein Kinase: p38MAPK Signalling Pathways and their Role in Human Diseases

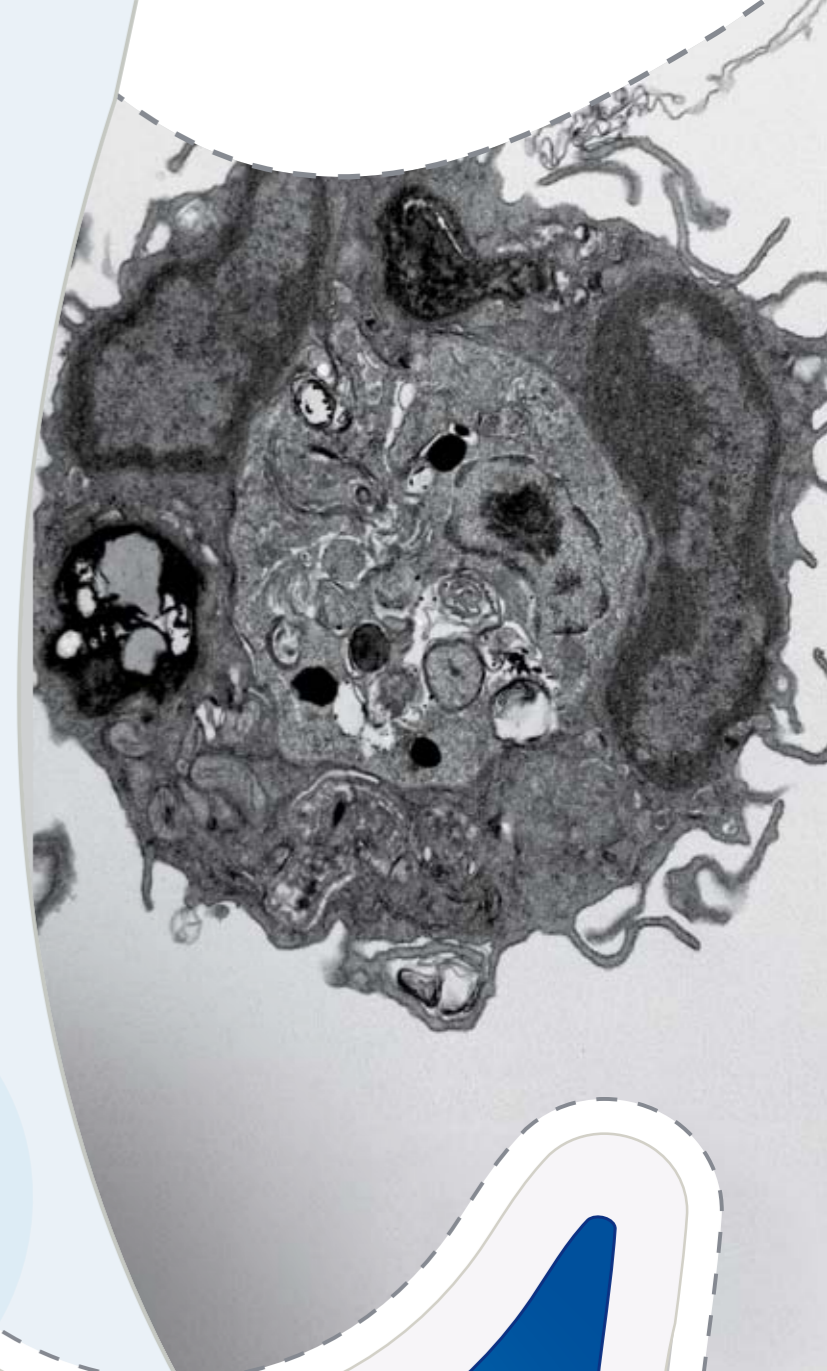
The aim of our group is both to discover how members of the p38MAPK family regulate cell function in physiological conditions and in response to environmental stresses, infection and proinflammatory cytokines, and to understand how they are deregulated in several human disease situations such as oncogenic transformation and inflammation.



Our research is focused on (1) the discovery of new substrates, interacting proteins and inhibitors for these kinases, and the study of their physiological roles using transgenic mice for the different p38 isoforms, and (2) the study of p38MAPK as a link between chronic inflammation and cancer, and as mediators of chronic inflammatory diseases. These studies utilize biochemical, cell biology as well as whole animal model approaches.

There are four members of the p38MAPK family (p38 α , p38 β , p38 γ and p38 δ), which are similar in amino acid sequence but differ in expression patterns, substrate specificities and sensitivities to inhibitors. In recent years, our group has centred on elucidating the regulation and roles of the p38MAPK family members p38 γ and p38 δ . We found that p38 γ interacts and is the physiological kinase of several PDZ domain-containing proteins. In particular, p38 γ interacts with and phosphorylates the tumour suppressor protein hDlg, regulating its association to the cytoskeleton and to nuclear protein-RNA complexes.

We are currently studying how p38 γ regulates the integrity of nuclear and intercellular-junctional complexes, cell adhesion, migration and polarity, as well as cell cycle and proliferation in response to many kinds of external stimuli. A large body of evidence indicated that p38 α MAPK activity is critical for production of proinflammatory cytokines, whose uncontrolled production is a major cause of chronic inflammatory diseases. Nonetheless, little is known about the role of p38 γ and p38 δ isoforms in these processes. We are currently undertaking further studies to study this, as well as their role in the development of cancer associated to inflammation, using the genetically modified mice we have generated.



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Inmunology Oncology

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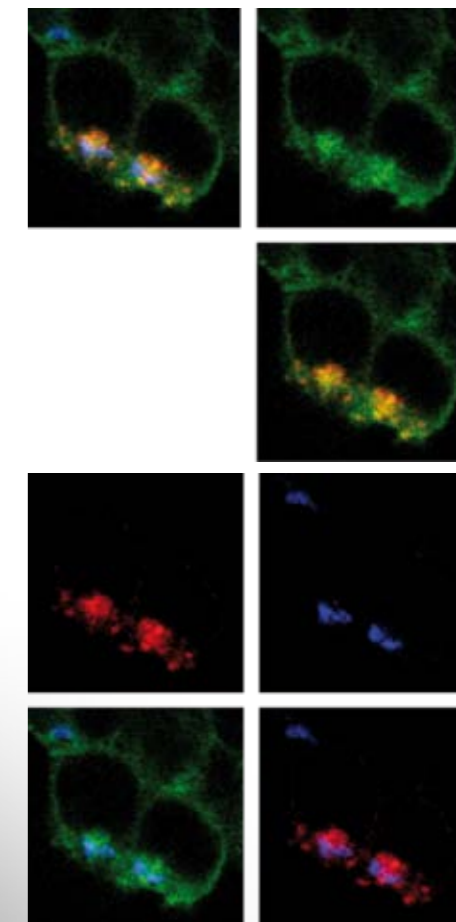
Chemokine-Mediated Cell Migration and Endocytosis

Chemokines are chemotactic cytokines that act through plasma membrane-tethered receptors.

They are involved in cell functions that include regulation of immune defense, as well as in tumor growth, atherosclerosis and asthma. Migration of immune cells from the blood into tissues is crucial in immune surveillance and host defense. In our group, we investigate the chemokine response of monocytic cells using cell biology and knock out animal models.

Cell migration is a complex biological function triggered by integrins, growth factors and chemokines. In order to evaluate the importance of membrane trafficking during cell migration towards a chemokine gradient, we monitored the number of the chemokine receptor CCR2 present at the cell surface. We found that cells that did not migrate needed higher concentrations of the chemokine CCL2 for the CCR2 internalization to occur. These data suggest that internalization occurs during chemotaxis and that non-migrating cells exhibit a reduced internalization. We manipulated clathrin/dynamin-mediated endocytosis to understand the molecular mechanisms implicated in CCL2-stimulated cell migration. Sucrose treatment of cells is described to interfere with endocytosis mediated by clathrin, a protein involved in transfer of material between cell organelles. We found that sucrose treatment impaired CCR2B internalization and migration of monocytic cells. When RNA interference was used to knock down clathrin, CCR2B internalization and transferrin uptake were prevented, as was cell migration. Dynamin is an endocytic pinchase implicated in the scission of vesicles. This function can be inhibited with the help of dynasore. When cells were treated with dynasore, we observed a reduction in cell migration. Our findings indicate that hindering membrane trafficking reflects in deficient cell migration. The relevance of membrane trafficking in the process of cell migration needs further evaluation using model of chemokinesis (wound assay) and live imaging techniques.

Cells expressing CCR2BmCherry (red) were CCL2-treated (150 nM; 5 min) and incubated on ice, then formaldehyde-fixed. Cells were labeled with antibodies to vesicular marker clathrin (green), the Golgi marker giantin (blue), followed by goat anti-rabbit AMCA and anti-mouse Alexa 488 labeled secondary antibodies. Images were obtained by confocal microscopy. Colocalization of receptor and clathrin is indicated (yellow), receptor and giantin (magenta) and clathrin and giantin (cyan). First panel represents the three labels. No overlap (white) was detected.



We have also found that the nuclear receptor RXR (Retinoid X Receptor) regulates the transcription of the chemokines CCL6 and CCL9 in vivo and in vitro. In the blood of mice lacking RXR in macrophages, we found lower levels of CCL6 and CCL9. When these mice suffer from peritonitis, they showed less inflammation and fewer leukocytes were attracted. In addition, these mice were less prone to sepsis and therefore our results put forward RXR as a target for treatment of inflammatory processes.

Our studies established CCR2 endocytic routes and their importance in monocytic cell migration. These insights into the molecular mechanisms of desensitization and signaling define potential targets for therapeutic intervention in diseases such as asthma and allergic inflammation.

In another collaborative work, we devised a method that assigns the topology of the membrane proteins using fluorescent proteins (FP). This so-called pH exchange assay (PEA) takes advantage of the pH sensitivity of GFP and YFP. Major advantages of the PEA are its technical simplicity, since it requires only imaging on a widefield or confocal microscope, its applicability to live, undisturbed cells, and the ability to quantify the proportion of inside/outside orientation for proteins with multiple topologies. We believe that this assay will be of interest to many groups working with membrane proteins.

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Inhibition of dynamin prevents CCL2-mediated endocytosis of CCR2 and activation of ERK1/2. M.A. García-Lopez, A. Aguado-Martínez, C. Lamaze, C. Martínez-A. and T. Fischer (2009). *Cell Signal.* 21:1748.

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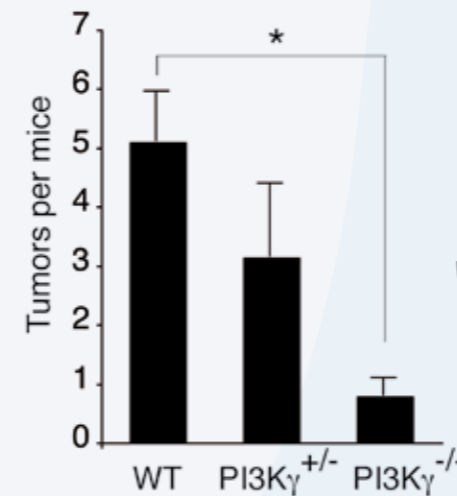
The Role of Ras Effectors in Inflammation and Cancer

The Ras GTPase is mutated in approximately 15% of human tumours, and these mutations are especially frequent in lung, colon and pancreatic carcinomas.

In normal cells, Ras participates in several biological responses, regulating cell cycle progression, migration, differentiation and cell survival as well as immune system development and function. Ras mediates these functions through the activation of three effector pathways, Raf/MAPK, PI3K and the Ral GTPases. The focus of our research is to analyse the contribution of these Ras effectors to several aspects of inflammation and tumour development.

We analyse the role of Ral GTPases in the immune system using genetically modified mice and biochemical tools to knock down the expression of these GTPases. In cytotoxic cells, both Ral isoforms, RalA and RalB, are activated rapidly after target cell recognition, and translocate to the cell-cell contact zone (Fig. 1). A critical step in cell-mediated cytotoxicity is the directed secretion of lytic granules at the immunological synapse, by which lytic molecules are delivered specifically to the target cell, and neighbouring cells are protected from damage. Ral GTPases have been shown to participate in polarized secretion in different cell types; we therefore study Ral function in the regulation of cell-mediated cytotoxicity.

A large body of evidence now supports a correlation between inflammation and cancer, although the molecular mechanisms that govern this process are poorly understood. This correlation is particularly clear in patients with inflammatory bowel disease, who have an increased risk of developing colorectal cancer. We use a murine model of inflammatory bowel disease to dissect the contribution of a PI3K isoform, PI3K γ , to inflammation-associated colorectal cancer. We found that PI3K γ -deficient mice had lower



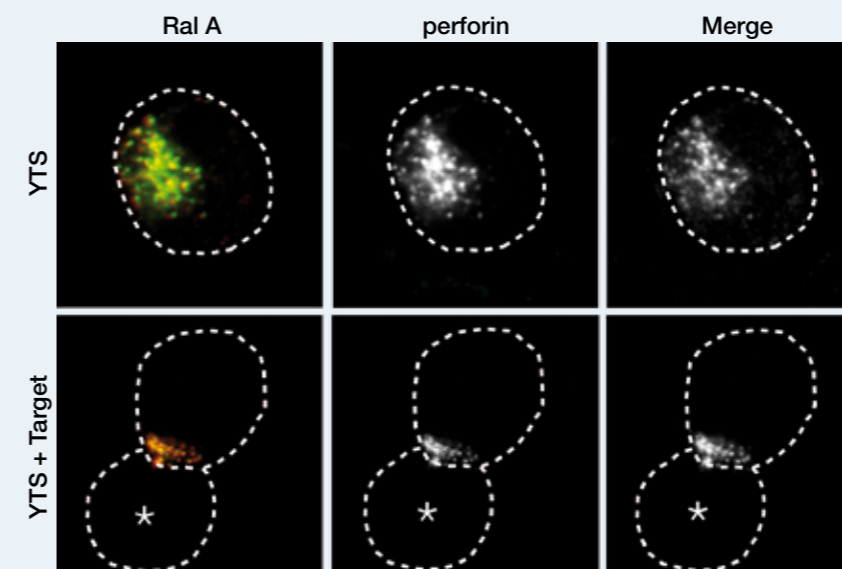
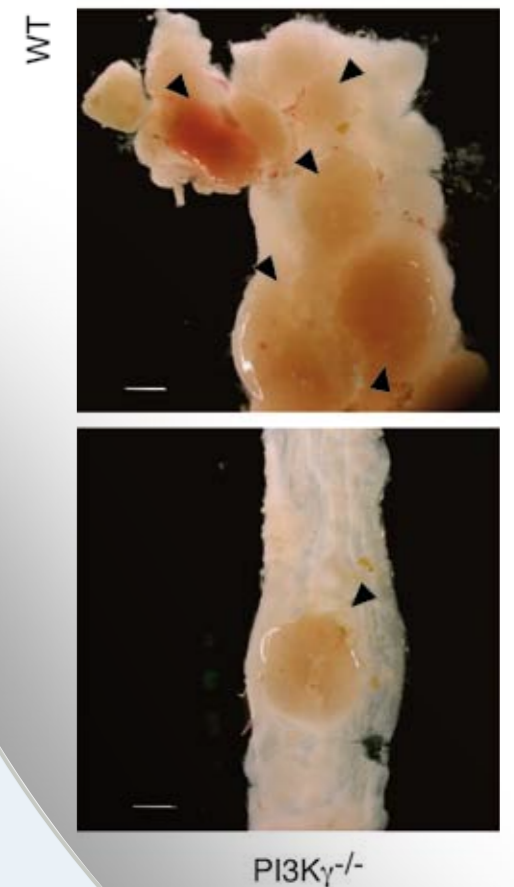
incidence and multiplicity of tumours than control mice (Fig. 2). PI3K γ ^{-/-} animals also showed a reduction in colon inflammation levels, with defective activation and infiltration of myeloid cells and, in consequence, defective recruitment of T cells to the colon. These data suggest that PI3K γ ameliorates inflammation-associated cancer by modulating the immune response.

By understanding the molecular mechanisms that regulate different steps during tumour formation, we can contribute to finding potential targets for the development of new cancer treatment therapies.

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González-García A, Sánchez-Ruiz J, Flores JM, Carrera AC (2010) Phosphoinositide 3-kinase γ inhibition ameliorates inflammation and tumor growth in a model of colitis-associated cancer. *Gastroenterology* 138:1374-1383.

Representative images of colons from WT and PI3K γ ^{-/-} mice showing tumours in the distal region. Quantification of tumour multiplicity is shown (right).



Ral localise to lytic granules in YTS cells. YTS cells were incubated alone or with 721.221 target cells at a 1:4 ratio (20 min), fixed, stained with anti-RalA (green) and anti-perforin (red) and analysed by confocal microscopy. *denotes 721.221 target cells.



Inmunology
Oncology

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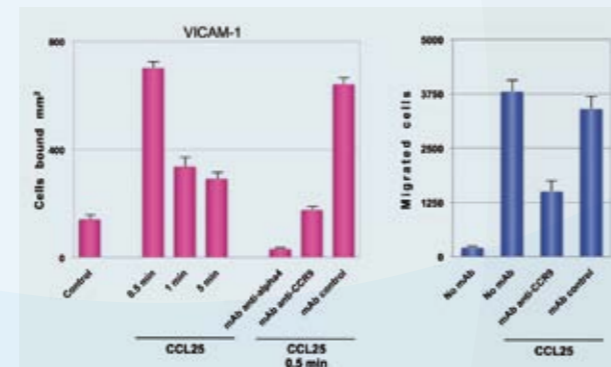
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Left, CCL25 stimulates the T cell adhesion mediated by $\alpha 4\beta 1$ integrin. BCECF-AM-labeled MOLT-4 T cells were treated with anti- $\alpha 4$, anti-human CCR9 or control mAb, incubated for the stated times alone or with soluble human CCL25, and tested in adhesion assays to VCAM-1. Right, Anti-human CCR9 mAb blocks migration of MOLT-4 T cells in response to CCL25. Chemotaxis assays were performed in transwell chambers, control or mAb-treated cells were added to the transwell inserts and placed in wells containing 200 nM CCL25. Following a 2 h incubation, cells migrating through the membranes into the lower wells were harvested and counted by flow cytometry.

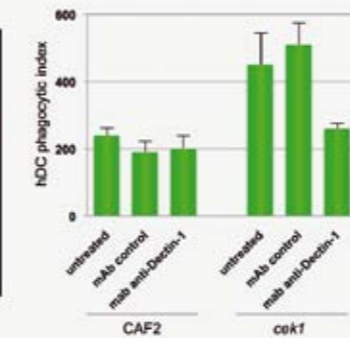
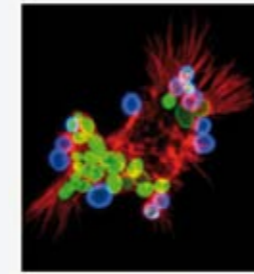
Chemokine Receptors in
Cancer Biology

Our main research interest is to understand how chemokines mediate the interaction between tumor cells and their microenvironment, and how they participate in the control of tumor growth and progression.

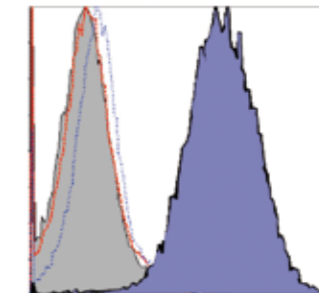
We study the contribution of chemokine receptors to cancer cell physiology, and evaluate their potential as antitumor targets.



Chemokines are small proteins that bind to G protein-coupled receptors and regulate leukocyte migration during homeostasis, inflammation and infection. Chemokine action extend to all cell types, however, and appears to be important in defining organ-specific metastatic destinations during cancer spread. In some solid tumors, levels of the chemokine receptors CCR7 and CXCR4 correlate with cell dissemination in lymph nodes or other sites; CCR6 and CX3CR1 expression is related to cancer cell homing to specific organs, suggesting chemokine receptors as promising therapeutic targets. The chemokine receptor CCR9 is expressed primarily on thymocytes and in a small subset of intraepithelial lymphocytes; its only natural ligand, CCL25, is expressed mainly in thymus and the small bowel. Recent reports showed that CCR9 overexpression increases the migratory and invasive capacity of



Left, phagocytosis and killing of *Candida albicans* by human monocyte-derived dendritic cells (hDC). Confocal microscopy images of dendritic cells showing extracellular yeast cells (blue), internalized yeast cells (green), and actin (red). Right, the fungal phagocytic indices of hDC exposed to wt CAF2 and the *cek1* deletion mutant were measured by differential immunofluorescence labeling. The phagocytic index was assessed by counting the number of internalized yeast cells per 100 phagocytes, after nontreatment or treatment of primary cells with isotype control mAb or the anti-dectin-1 MGD3 mAb.



Anti CCR9 mAb recognize specifically the CCR9 chemokine receptor in stably transfected HEK 293 cells. Human CCR4, CCR5, CCR9 or empty vector transfected cells were stained with anti-hCCR9 mAb, followed by PE-conjugated anti-mouse immunoglobulin antibodies and analyzed by flow cytometry.

prostate cancer cells, directs ovarian cancer and melanoma cell metastases to the small intestine, and increases proliferation of and resistance to apoptosis by acute lymphoblastic leukemia-derived cell lines. CCR9-mediated intracellular signaling activates the anti-apoptotic PI3K, Akt, PTEN, mTOR, ERK1/2 and GSK-3 β pathways, and downregulates activation of caspase-3, leading to survival and increased cell proliferation.

Our group is focused in determining the role of CCR9 in tumor physiopathology. We have generated anti-human CCR9 monoclonal antibodies (mAb) to characterize and follow CCR9 expression by flow cytometry, Western blot and immunomicroscopy, and for blocking receptor signaling. Our neutralizing anti-CCR9 mAb are being used to evaluate the CCR9 participation in tumor cell cycling, survival, migratory and invasiveness using human tumor lines, and in tumor progression and metastasis in xenogeneic mouse models. We use quantitative real-time PCR and immunohistochemistry to determine whether antibody action produces changes in mRNA and protein levels. CCR9-expressing human carcinoma cell lines and RNA interference approaches are also used to study the molecular mechanisms that underlie CCR9-mediated effects. In addition, we will test the usefulness of these mAb for diagnostic imaging and for antagonist screening of low molecular weight compounds for drug development.

We work in collaboration with Drs. Gabriel Márquez (Genetrix), Laura Carramolino (CNIC), Elena Fernández-Ruiz (Hospital La Princesa, Madrid), Joaquín Teixido (CIB/CSIC), Julio Gutiérrez, Ricardo Villares and Carlos Martínez-A (CNB/CSIC).

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Inmunology
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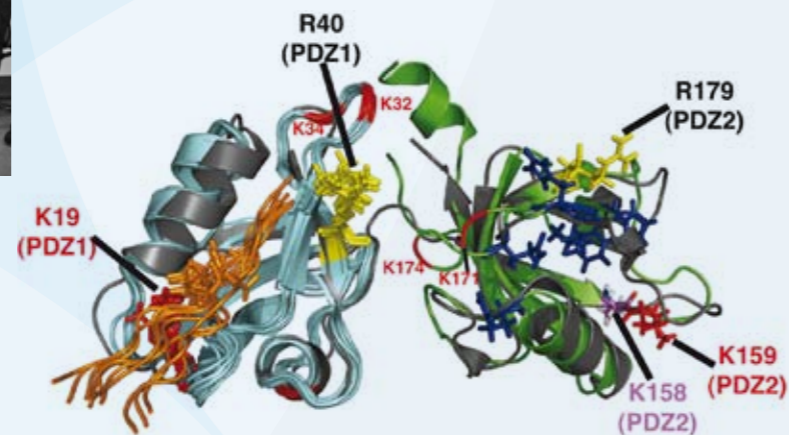


Signalling Networks in Inflammation and Cancer

Inflammation is a complex stereotypical response that is essential for effective defence of the organism against harmful stimuli such as pathogens, irritants or tissue damage.

Discovery of the detailed processes of inflammation has revealed a close relationship between the inflammatory reaction and the immune response. Indeed, a hallmark of inflammation is the directed migration (chemotaxis) of inflammatory cells (mostly leukocytes) through blood vessel walls to the site of injury. Once wound healing is complete, inflammation resolves and tissue homeostasis returns. Nonetheless, a deregulated response to tissue damage might lead to autoimmunity and chronic inflammatory diseases, and can also promote cancer.

Recent clinical and experimental evidence indicates that solid tumours exacerbate inflammation to promote their own progression. This leads to a tumour microenvironment largely orchestrated by inflammatory cells, altering the metabolic needs of the tissue and fostering neoangiogenesis, proliferation, survival, mutagenesis, migration and metastasis of malignant cells. Tumour-induced inflammation usually leads to immunosuppression, impeding the immune system surveillance function and clearance of the tumour; indeed, breaking immunosuppression has been demonstrated as a useful, efficient way to eradicate cancers. Immune cells might therefore provide both anti- and protumorigenic signals, which could be harnessed or attacked for therapeutic purposes.



We aim to identify and understand key molecules/pathways responsible for the aberrant inflammatory reaction involved in the development or outcome of inflammation-associated pathologies. Our research projects focus on distinct steps of this reaction:

- (i) Study at the cellular level of key signalling pathways that regulate acquisition of a motile phenotype in leukocytes
- (ii) Understand the role of specific chemokines/chemokine receptors in orchestrating the activation of the adaptive immune response
- (iii) Identify key regulators of terminal differentiation in innate immune cells within the inflammatory environment
- (iv) Examine the relevance of the vascular system in controlling inflammatory migration of specific leukocyte subtypes

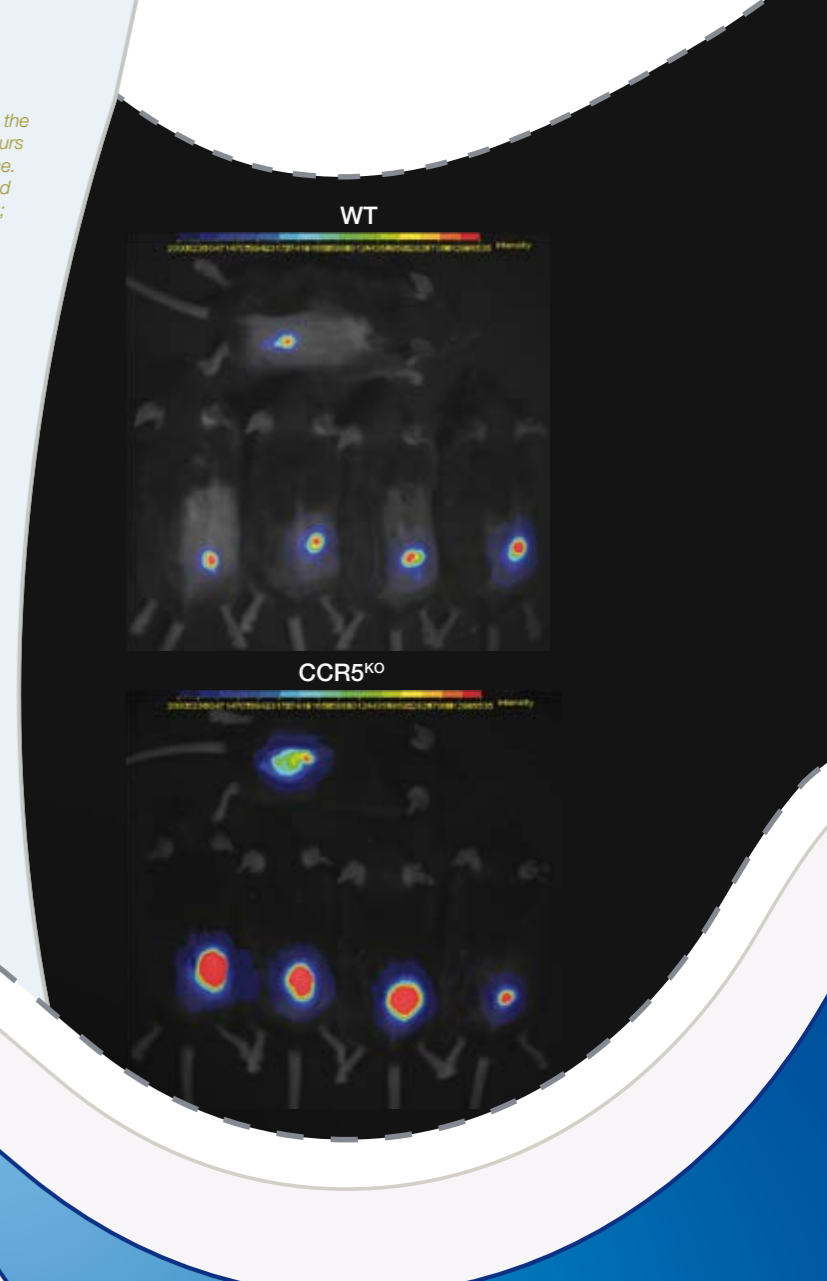
We hope to understand critical cellular, molecular, and chemical mediators through which tumours promote inflammation and subvert the immune system to favour their progression. Comprehension of the mechanisms that balance pro- and anti-tumour immunity could lead to the design of more effective anti-cancer therapeutics.

Although the major focus of our research is the inflammatory reaction to cancer, we are also interested in understanding the chronic inflammation associated to autoimmune diseases.

Legend:
 ■ syntein ■ EBP50-PDZ1 Sol. 1 ■ EBP50-PDZ2
 ■ PIP5Kβ C-terminal peptide

Model for the binding of the PDZ-binding motif identified in the Type1 PIP5K-beta isoform, with the tandem EBP50-PDZ domains, based on syntein structure. It is indicated the critical residues on EBP50 involved in binding to PIP5K.

Bioluminescence images showing the differential growth of pancreatic tumours in wild-type and CCR5-deficient mice. Luciferase activity was measured and transformed to a pseudocoloured image; red represents maximal light intensity.



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**Inmunology
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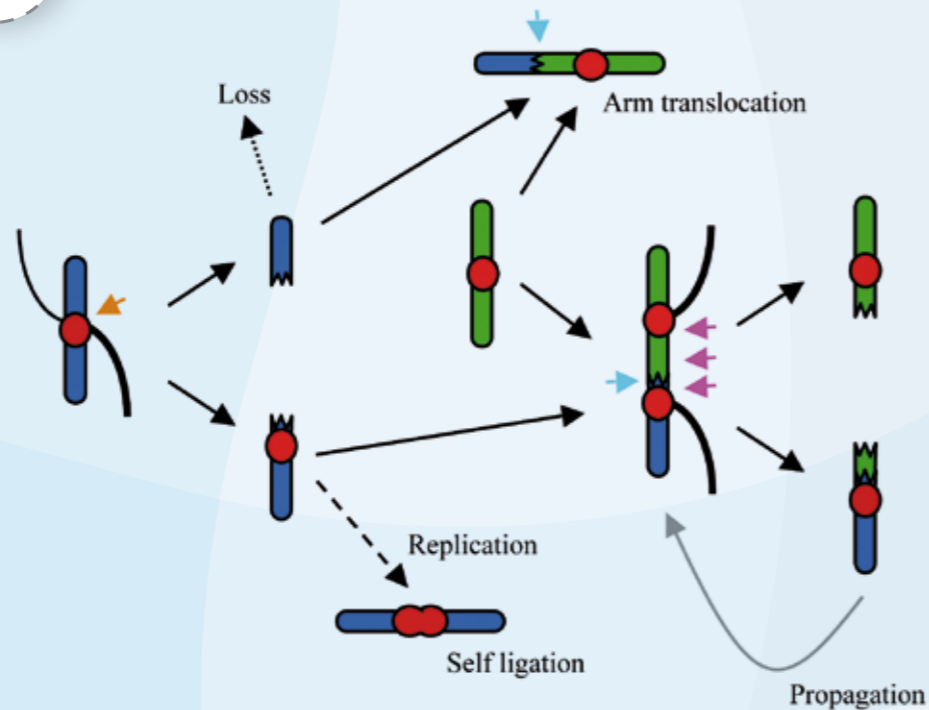
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Linking Centrosomal Aberrations to Chromosomal Instability

Our group studies the link between cell division and genomic instability, with a special interest in the death inducer obliterator (Dido) protein family.



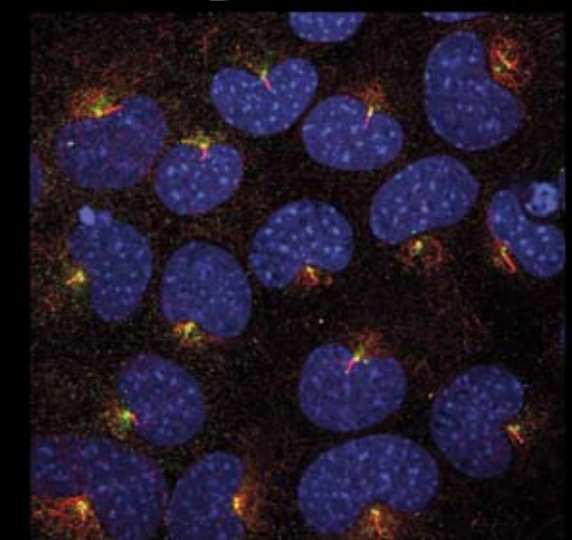
Model of centromere fission in chromosomal instability.

After Dido3 was found in the nucleus and centromeres from mitotic cells some years ago, we recently showed that Dido3 is associated with the synaptonemal complex in meiosis [1]. This observation points at a general role for Dido3 in cell division and chromosome maintenance. The localization of Dido3 in the synaptonemal complex is regulated by epigenetic modifications that are recognized by a histone-interacting domain in the protein's aminoter-

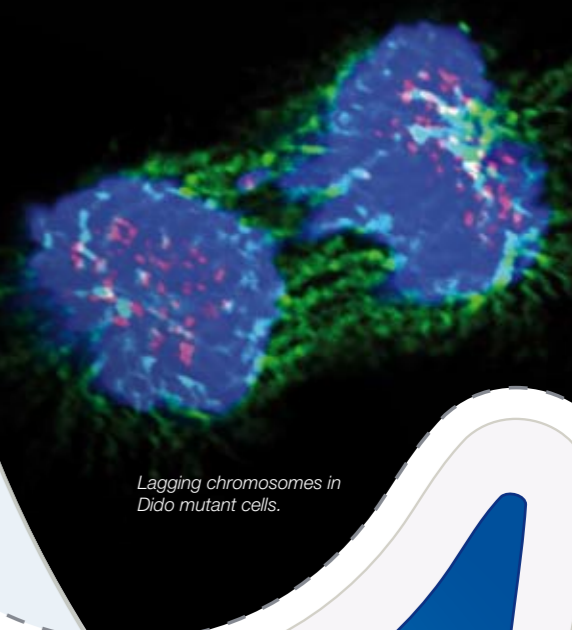
minus. A similar mechanism might govern the distribution of Dido3 between the nucleus and centromeres in mitotic cells. After having identified of the determinants for Dido3 localization, we plan to elucidate the mechanism by which Dido3 regulates target proteins in the centrosome and synaptonemal complex.

In addition to the role of Dido proteins in cell division, we are interested in the origins of genomic instability in sporadic carcinomas. The most common type of genomic instability in solid tumors is chromosomal instability (CIN), in which chromosome number changes occur together with segmental defects. This means that changes involving intact chromosomes accompany breakage-induced alterations in CIN tumors. Whereas numerical alterations are attributed to chromosome missegregation, the origins of breakage in CIN tumors remain disputed. Recently, we proposed a model of chromosome breakage based on spindle defects and kinetochore distortion [2]. Using *Dido* gene mutants, we obtained the first evidence of spindle-generated chromosome breakage [3]. Our data showed that reduced control over the mitotic spindle not only causes losses and gains of intact chromosomes, but also produces kinetochore distortion and shearing of kinetochore-associated chromatin.

Spindle-controlled shearing of kinetochore-associated chromatin, a phenomenon we termed centromere fission, occurs during mitotic chromosome segregation. Broken chromosomes generated by centromere fission are therefore separated from their counterparts, frequently end up in micronuclei, and are repaired when recombination is nearly inactive. As a consequence, chromosome arms containing centromeric breaks often fuse to healthy chromosomes, in a process that depends on non-homologous end joining [4]. A classification of genetic aberrations showed that centromere fission and capture of healthy chromosomes is the mechanism that best explains genomic instability in sporadic carcinomas. Our future work on chromosomal instability will be directed at elucidating the details of the mechanisms that underlie centromere fission and the identification of centromeric fusion products in cancer cells.



Ciliogenesis in *Dido* mutant cells.



Lagging chromosomes in *Dido* mutant cells.

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Inmunology
Oncology

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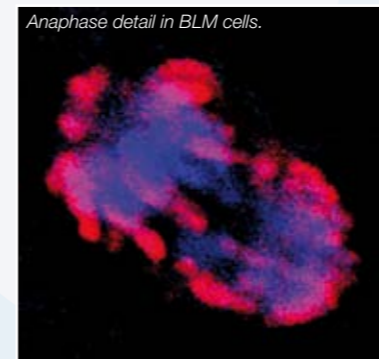
Chemokine Receptors: New Targets for Therapeutic Intervention

Since the first reports on chemokine function, much information has been generated on the implications of these molecules in numerous physiological and pathological processes, as well as on the signaling events activated through their binding to receptors.

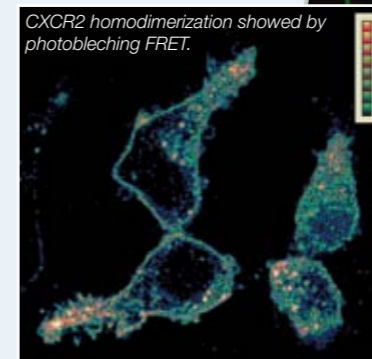
Despite these extensive studies, no chemokine-related drugs have yet been approved for use in patients with inflammatory or autoimmune diseases. This discrepancy between efforts and results has forced a re-evaluation of the chemokine field.

Using classic biochemical techniques and new methodologies based on energy transfer between fluorophores (FRET), we have explored chemokine receptor conformations at the cell surface and found that, as is the case for other G protein-coupled receptors, chemokine receptors are not isolated entities that are activated following ligand binding; rather, they are found as dimers and/or higher order oligomers at the cell surface, even in the absence of ligands. These complexes form organized arrays that can be modified by receptor expression and ligand levels, indicating that they are dynamic structures.

Clusters of chemokine receptors are expressed at the cell surface. It is thus plausible that receptor dimers organize in such clusters, like bundles of cigars. Ligands then modulate and stabilize specific receptor conformation to trigger functional responses without disrupting cell surface receptor arrays. Ligand-mediated internalization of a given receptor pair does not necessarily alter the levels of other receptors in the 'bundle'. It is nonetheless possible that the conformation of resting receptors in an array might be affected by ligand binding to a responding receptor. Such 'allosteric' conformational changes might not be restricted



Anaphase detail in BLM cells.



CXCR2 homodimerization showed by photobleaching FRET.

Neural stem movement.

to neighboring dimers, but might extend through the array in a domino effect.

The chemokines also activate a tyrosine kinase pathway that shares many components with the biochemical pathway activated by the cytokine receptors. We have reported that chemokines activate the JANUS kinases (JAK), which associate to the chemokine receptor and promote its rapid tyrosine phosphorylation. Through the JAK/STAT pathway, the chemokines trigger suppressor of cytokine signaling (SOCS) expression. The SOCS intracellular proteins are thus key physiological regulators of cytokine and chemokine responses, SOCS proteins regulate signal transduction by binding directly to JAK or by competing with STAT for the phosphorylated receptor; in addition, they target ubiquitinated signaling intermediates for degradation by the proteasome pathway through ECS (elongin-Cullin-SOCS) E3 ligase formation. As consequence of this last effect, SOCS1 function as a tumor suppressor blocking cell cycling in human melanoma by affecting G1/S and mitosis.

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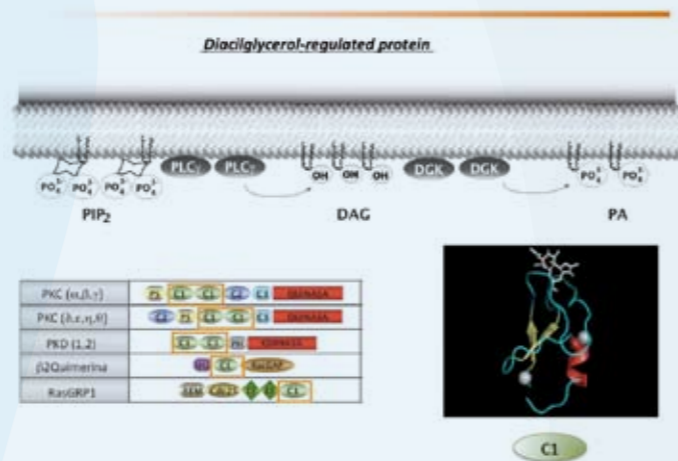
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Lipid Signalling

Diaclycerol (DAG) is a lipid with unique functions as a basic membrane component, as a lipid metabolism intermediate and as a signaling molecule.



DAG regulates C1 containing proteins. Top. DAG metabolism at the membrane. Bottom left. C1 domain containing proteins. Bottom right. Ribbon model of PLCdelta C1 domain

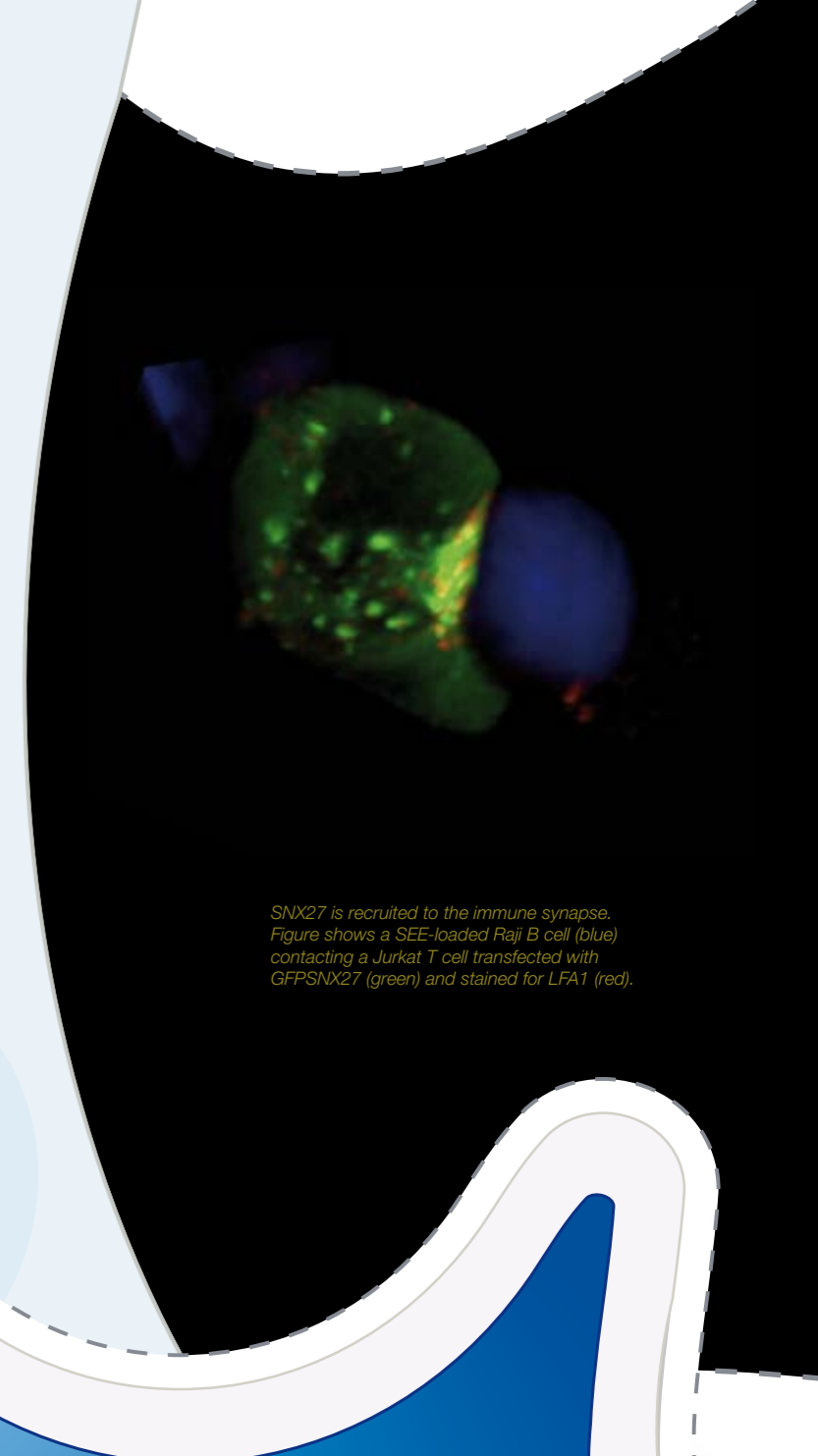
In eukaryotes, a host of proteins have evolved the ability to bind to DAG and are thus modulated by this lipid, creating additional levels of control to meet the complex needs of multicellular organisms. DAG-regulated proteins participate in neuronal and vascular patterning, synapse transmission, glucose transport and are critical for the correct immune response. Altered DAG functions are related to transplant rejection, inflammation, diabetes, allergy and autoimmune disease. Sustained DAG generation, on the other hand, is associated with malignant transformation. The complexity of DAG-regulated processes emphasizes the need for studies aimed to evaluate the potential therapeutic manipulation of DAG generation and clearance.

Our group studies the contribution of DAG-regulated mechanisms to T cell activation and oncogenic transforma-

tion, so that steps of these processes can be manipulated for possible therapeutic benefit. Our final goal is to demonstrate that modification of DAG metabolism represents a novel and understudied strategy directed to the management of a more effective immune response and/or treatment of cancer.

We investigate the mechanisms by which DAG binds and regulate C1 domain-containing proteins. In addition to the well characterized PKC family, vertebrates express six additional families of DAG regulated proteins: chimaerins, DGK (beta and gamma), PKD, Munc13, RasGRP and MRCK. The specific expression in T cells of C1-containing GEFS and GAPS for small GTPases of the Ras and Rho family has uncovered new and strategic functions for DAG in the regulation of Ras and Rac. We study the spatial generation of DAG using fluorescent DAG sensors. Studies from our laboratory have also provided new insight into the mechanisms that govern DAG-mediated regulation of RasGRP1 and β2 chimaerin during T cell activation.

Another important area is the study of Diacylglycerol kinases (DGK), which transform DAG into phosphatidic acid, and represent important modulators of DAG-dependent functions. We use biochemical and genetic approaches to better understand how antigen mediated stimulation determines membrane localization/activation of DGKα and ζα, their site of activation and the nature of the interacting partners. We also explore the role of these isoforms in the maintenance of transformed state through regulation of the PI3K/MTOR pathway. We expect that our findings will contribute to assess the therapeutic potential of the DGK enzyme family as tools for a better and more effective management of the immune response and treatment of cancer.



SNX27 is recruited to the immune synapse. Figure shows a SEE-loaded Raji B cell (blue) contacting a Jurkat T cell transfected with GFP-SNX27 (green) and stained for LFA1 (red).

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**Inmunology
Oncology**

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**Function of the
c-Myc Proto-Oncogene
*in vivo***

At present, with 1.7 million deaths each year, cancer represents the second most important cause of death in Europe.

In up to 50% of all human cancers, constitutively enhanced expression of proto-oncogenes of the *myc* family is a characteristic signature. *Myc* deregulation is due to rearrangements or other mutations in either one of the three *myc* genes. Myc proteins are members of a basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor family (N-, L- and c-Myc) that can either activate or repress expression of their target genes. The study of Myc function is a complex task. Myc members have been shown to bind to several thousand loci in the genome of humans and mice and regulate a variety of different genes. This large number of Myc targets affects a wide range of biological processes such as cell cycle control, apoptosis, protein synthesis, energetic metabolism, senescence, cell polarity and cell differentiation, which all are known to play a role in human cancer development. However, it remains to be determined which of these functions are the most relevant in Myc-dependent tumorigenesis. Among all these Myc functions, the role of c-Myc in cell differentiation is poorly understood. In this context, our group is interested in understanding the molecular mechanisms that mediate the action of the proto-oncogene *c-myc* in cell differentiation. With this aim in mind, we have focused our efforts to address this question in a well-defined setting *in vivo* such as B lymphocyte differentiation. In recent years, we have developed several conditional mouse models to inactivate c-Myc at different developmental stages in B lymphocytes. These models have proved to be very useful to place c-Myc in the context of the transcription factors necessary for B lymphocyte differentiation. Finally, we expect to translate all this knowledge to pathological situations caused by deregulation of c-Myc expression in B lymphocytes.

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Immunofluorescence staining of spleen 10µM cryosections from c-mycfl/fl or c-mycfl/+;cd19cre/+;rosa26egfp/egfp mice, 10 days after TNP-KLH immunization. Sections were stained with biotinylated PNA developed with avidin-Cy3, to allow identification of germinal centers. Magnification is 600x for the homozygous sample and 200x for the heterozygous.



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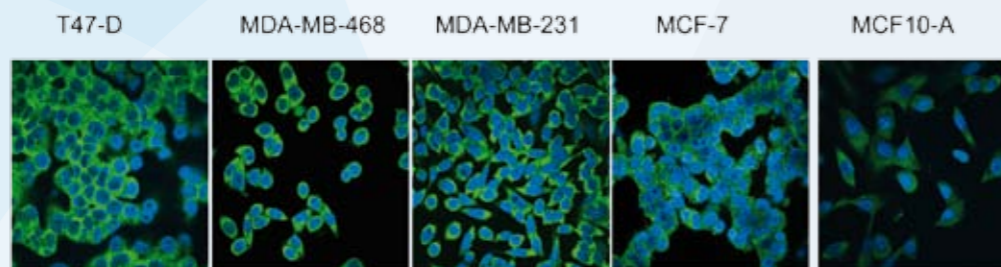
Function and Regulation of APRIL, a TNF Protein: Implications in Pathology

We focus on the study of APRIL (a proliferation-inducing ligand), a member of the TNF family proteins named for its ability to stimulate the proliferation of tumour cells *in vitro*.

APRIL binds to two known TNF receptors, TACI and BCMA. In addition, APRIL binds to heparan sulphate proteoglycans (HSPG), although the biological significance is not yet clear. APRIL is expressed by several cell types (dendritic cells, macrophages, epithelial cells, osteoclasts) and secreted as a soluble factor.

APRIL is known to enhance B cell proliferation and cell survival; it also enhances T-independent humoral responses and promotes immunoglobulin class-switch recombination to IgG and IgA. Altered APRIL expression has been detected in pathological situations such as autoimmunity and cancer. We described that APRIL transgenic mice develop lymphoid tumours that originate from the expansion of peritoneal B cells. Tumours in these mice resemble human chronic lymphocytic leukaemia (CLL), and our analysis of CLL patient sera shows an increase in circulating APRIL levels that correlates with reduced overall survival. In these

Human breast cancer cells express APRIL protein. APRIL expression (green) and DAPI (blue) by confocal microscopy in a panel of breast cancer cell lines (MDA-MB213, MDA-MB468, T47D and MCF7) and control cells (MCF10A).

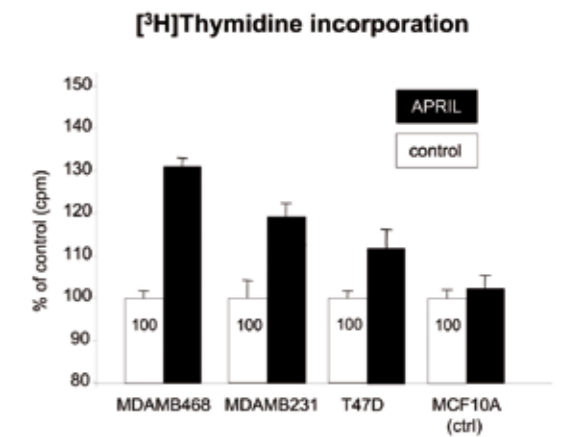


B cell malignancies, APRIL activates NFκB transcription factor, promotes tumour cell survival and protects cells from apoptosis.

Our main goal is to dissect APRIL function in the immune system and in pathological conditions. One research line studies the relevance of APRIL in epithelial breast cancer, using cell lines and mouse models. We detected high APRIL mRNA levels in 30% of the human primary breast tumours analysed and found that APRIL protein is expressed in various human breast cancer cell lines and promotes their proliferation. We identified molecules that stimulate APRIL secretion in these cancer cells and characterised the signalling transduction pathways activated by the cytokine. We also generated the MMTV-neu/APRIL double transgenic mouse and used syngeneic tumour transplant models in APRIL-Tg and APRIL-KO mice to study the influence of APRIL in epithelial tumour development *in vivo*. We are also exploring the potential clinical use of APRIL and analysing its expression in human solid primary tumours.

Another line of research uses mouse models and human samples to focus on autoimmune diseases (RA, SLE) and B cell deficiencies (XLA). We are examining the cell types responsible for APRIL/BAFF secretion and the factors that regulate it, the effects mediated by APRIL/BAFF, the target cells involved and the signalling pathways activated.

Finally, we are working on the generation of APRIL antagonist molecules that effectively block this cytokine as a strategy for blocking tumour development.



APRIL promotes proliferation of breast cancer cells. Control and cancer cells were cultured with APRIL (black) or alone (white) and a thymidine incorporation assay was performed. Graphic shows the percentages of proliferation normalized to the cpm of unstimulated cells (100%).

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PATENT

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**Inmunology
Oncology**

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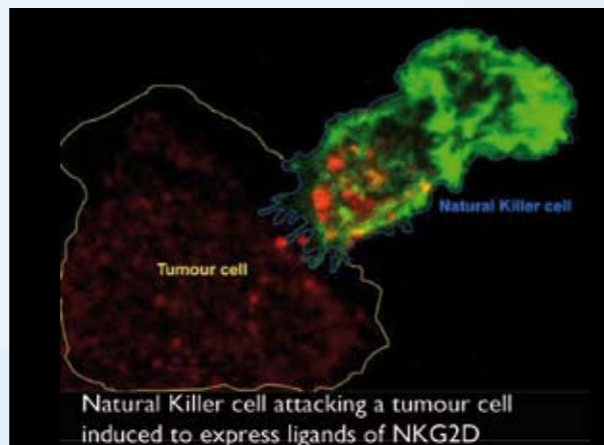
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**Receptor-ligand interactions
in immune responses to
cancer and viruses**

Current research in the laboratory addresses various issues related to the biology of NK cells and in particular the receptor NKG2D.

Some of topics of investigation represent the continuation of projects ongoing in the lab, before the move from Cambridge in October 2008, while others are new projects begun in the CNB:



Deposition of NKG2D containing lytic granules from an NK cell onto a tumour cell induced to express ligands of NKG2D.

Traffic and function of the NKG2D receptor

We have identified amino acids in the cytoplasmic tail of NKG2D and DAP10 that regulate internalisation of the receptor complex, and are now analysing how receptor recycling alters the threshold for signalling.

We have described how NKG2D/DAP10 receptor complexes polarise to the cytotoxic immune synapse in secretory lysosomes/lytic granules. We are now studying how the presence of this receptor complex in the lytic granules affects the fusion of the granules with the target cell membrane for delivery of the lethal hit.

We, and others, have described that chronic interactions with NKG2D-ligand-expressing target cells produces NK cell exhaustion/anergy. We are now analysing the molecular basis of this defect.

The use of human cytomegalovirus as a tool to study the regulation of expression of NKG2D ligands

We have a longstanding interest in studying the interactions between viruses and cells of the immune system as a strategy to gain insight into functionally important features of the immune system. We have noted that infection with human cytomegalovirus (HCMV) induces high levels of shedding of NKG2D ligands, and are characterising the biochemical basis of this effect *in vitro*. We have also gone on to show that the NK cells and CD8⁺ T cells of patients with active CMV-related disease have markedly reduced levels of NKG2D receptor expression *in vivo*, and are now working to understand the mechanisms underlying this phenomenon. These experiments are being done in collaboration with groups in the university teaching hospitals Gregorio Marañón and 12 de Octubre in Madrid, and we are beginning to collaborate with investigators in other regions of Spain.

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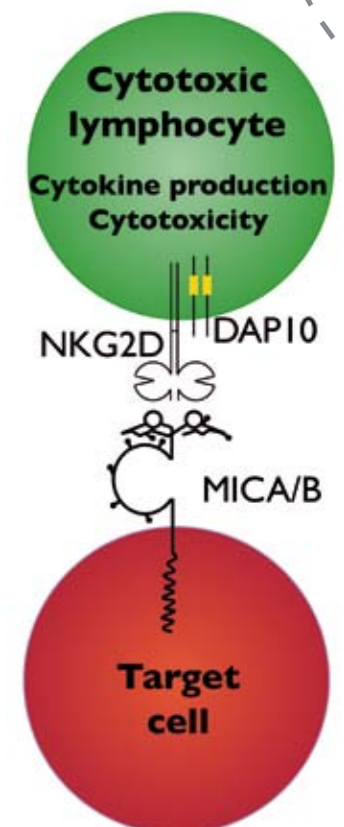
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The roles of NKG2D in regulation of the immune response

- 1) Immune surveillance of tumours
- 2) Autoimmune disease
e.g. Coeliac disease, rheumatoid arthritis, diabetes
- 3) Transplant rejection
- 4) Immune recognition of pathogens (e.g. HCMV, HIV)



Immunology
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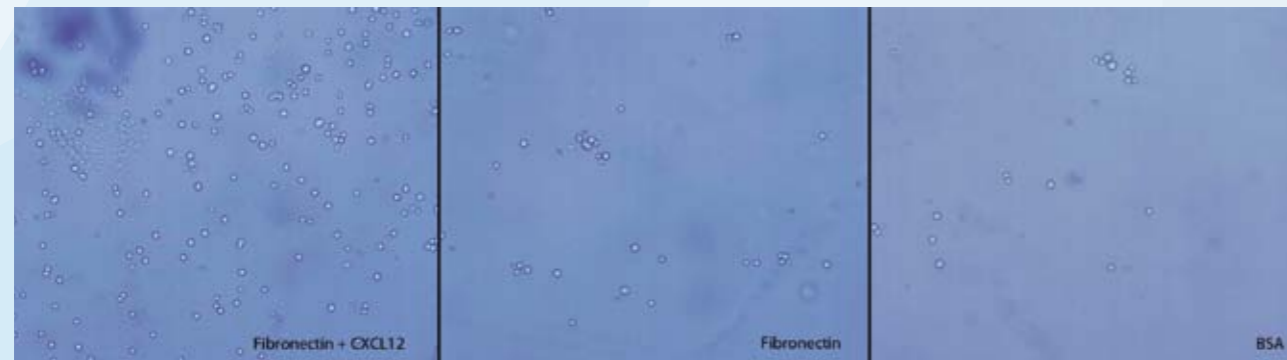
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Chemokine Signaling

Chemokines are a family of proinflammatory cytokines that, through interaction with seven transmembrane G protein-coupled receptors, play a key role in numerous biological processes, from organogenesis and leukocyte trafficking to host immune responses.

Signalling through chemokine receptors involves ligand-induced conformational changes in the receptor, allowing binding of JAK kinases and interaction with G proteins, initiating signalling cascades that lead to cytoskeletal rearrangement, gene expression, and receptor desensitization via internalization.

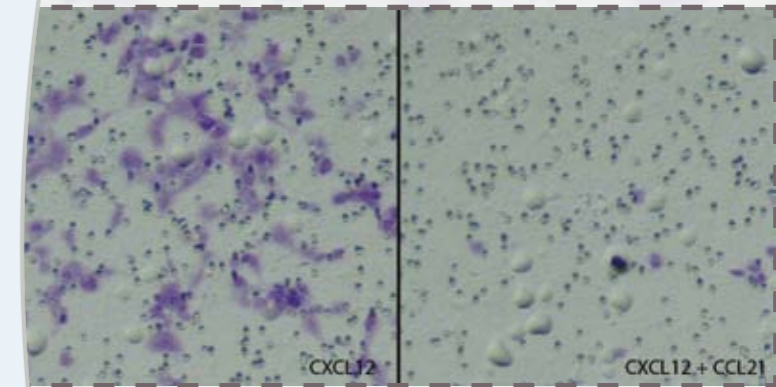


Melanoma cell adhesion to Fibronectin under flow conditions.

Chemokine receptors were thought to exist as isolated entities, allowing a 1:1 interaction with the corresponding ligand as the basic unit that triggers signalling and functional consequences. Chemokine biology is nonetheless more complex than initially predicted, as several studies suggest that chemokines can dimerise and that their receptors are found as dimers and/or higher order oligomers at the cell surface. Receptor oligomerisation might alter G protein specificity, coupling to signalling pathways, attenuate signalling, facilitating synergism between chemokine pairs and allowing GPCR crosstalk. The potential of chemokine receptor oligomerisation greatly increases the number of potential phenotypes, with implications for physiology and pharmacological intervention.

In recent years, we have studied chemokine-induced signalling pathways, including changes in receptor conformation, activation of tyrosine kinases and the various possibilities of chemokine signalling dependent on the cell microenvironment. Using the chemokine receptors CXCR4 and CCR7, we observed differences in coupling to distinct signalling pathways depending on the cell type analyzed. JAK kinases, G proteins and PI3 kinases are essential for some functions but dispensable for others. These effects are not only cell-type specific, but also chemokine receptor-specific; in some cases, there are also differences depending on the chemokine that activates a given receptor.

In previous work, we showed that the ligands for CXCR4 and CCR7 can couple to distinct signalling pathways in tumours and lymphoid cells. Our current research program is related to analysis of chemokine receptor conformations in different cell microenvironments. Using classical biochemical techniques and resonance energy transfer, we are analyzing interactions between chemokine receptors, which can indicate novel, specific activities triggered by chemokines. The main objective is to envisage new ways of modulating chemokine responses that could be therapeutically relevant by promoting or disrupting specific chemokine receptor complexes that indicate specific signalling events and therefore, cell-specific chemokine functions.



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T cell Signalling in Autoimmune Diseases and Cancer

The main goal of our group is to study the molecular mechanisms that regulate T cell signalling in the development of autoimmune diseases and cancer.

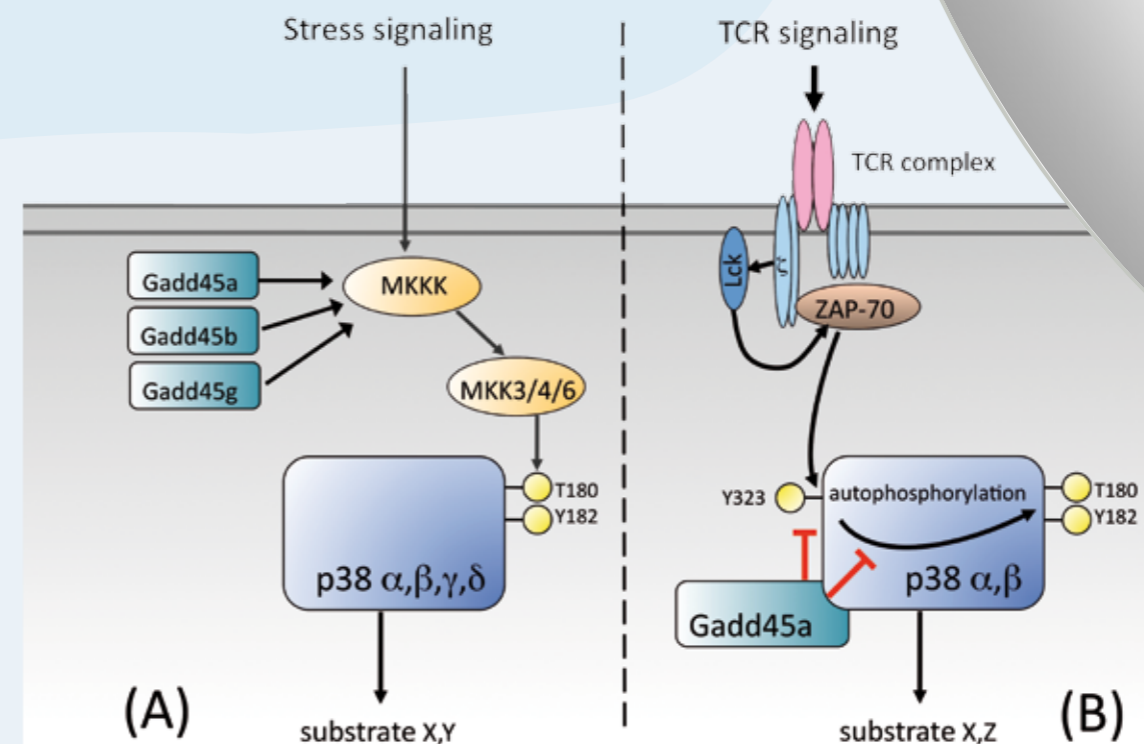
We focus on the role of the Gadd45 (growth arrest and DNA damage-inducible genes) and p38 MAPK (mitogen-activated protein kinase) families in suppression of autoimmunity and cancer. In mammalian cells, the best-characterised mechanism for p38 activation is via a phosphorylation cascade termed the classical MAPK pathway, in which a MAPK kinase kinase (MAPKKK) phosphorylates MKK3, MKK4 and MKK6. These kinases then phosphorylate p38 on the Thr180-Tyr182 motif, enhancing substrate access to the catalytic site and increasing its activity. We found that in T cells, p38 is activated by an alternative mechanism in response to antigen T cell receptor (TCR) signalling. TCR ligation increases activity of the Src kinase Lck, which phosphorylates Zap70. This tyrosine kinase phosphorylates p38 on Tyr323, which in turn induces autophosphorylation at Thr180, resulting in p38 activation. Gadd45a is a negative regulator of the alternative pathway. Gadd45a binds to p38, preventing Zap70-mediated p38 phosphorylation at Tyr323.

p38 MAPK is important in the pathogenic immune response in rheumatoid arthritis (RA). We addressed the function of the Tyr323-dependent and the classical MAPKK pathways in T cells from patients with distinct types of inflammatory arthropathies. We quantified phosphorylation of Tyr323p38 and (Thr180-Tyr182)p38 on T cells from healthy controls and patients with RA or ankylosing spondylitis (AS) to identify variables associated with p38 phosphorylation and disease activity.

In a cross-sectional study, we measured p38 phosphorylation on Tyr323 and Thr180-Tyr182 on T cells from 30 control individuals, 33 patients with AS, 30 with RA in remission and 79 with active RA. We collected clinical characteristics and

analysed the correlation between clinical variables, disease activity score (DAS) and p38 phosphorylation levels. Multivariate regression analysis was used to identify variables associated with p38 phosphorylation on Tyr323 and Thr180-Tyr182. We found that p38 phosphorylation on Tyr323 was higher in T cells from patients with active RA than in patients with RA in remission or with AS. Tyr323p38 phosphorylation was associated with disease activity as determined by the DAS28. Enhanced p38 phosphorylation was linked to Lck-mediated activation of the Tyr323-dependent pathway in the absence of upstream MAPKK activation (López-Santalla et al Arthritis Rheum, in press). Our results indicate that the Tyr323-dependent pathway has a central function in T cell-mediated p38 activation in RA patients and correlates with disease activity, suggesting selective inhibition of this pathway as an attractive target for specific downregulation of p38 activity in RA patients.

Gadd45a regulation of p38 activity in response to stress and TCR signalling. (A) In T cells, stress signals and cytokines induce dual phosphorylation of the Thr-X-Tyr motif (Thr180, Tyr182) and activation of all p38 MAPK isoforms, by triggering an upstream MAPK kinase kinase (MKKK) that activates MAPK kinases MKK3, 4 and 6. (B) Stimulation through the T cell receptor (TCR) activates Lck and ZAP70. ZAP70 phosphorylates p38 α and p38 β on the non-canonical Tyr323 residue; this induces autophosphorylation of Thr180 and p38 activation. Gadd45a binds p38 and blocks Tyr323 phosphorylation, Thr180 autophosphorylation, p38 activation, and alters p38 substrate specificity.



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**Inmunology
Oncology**

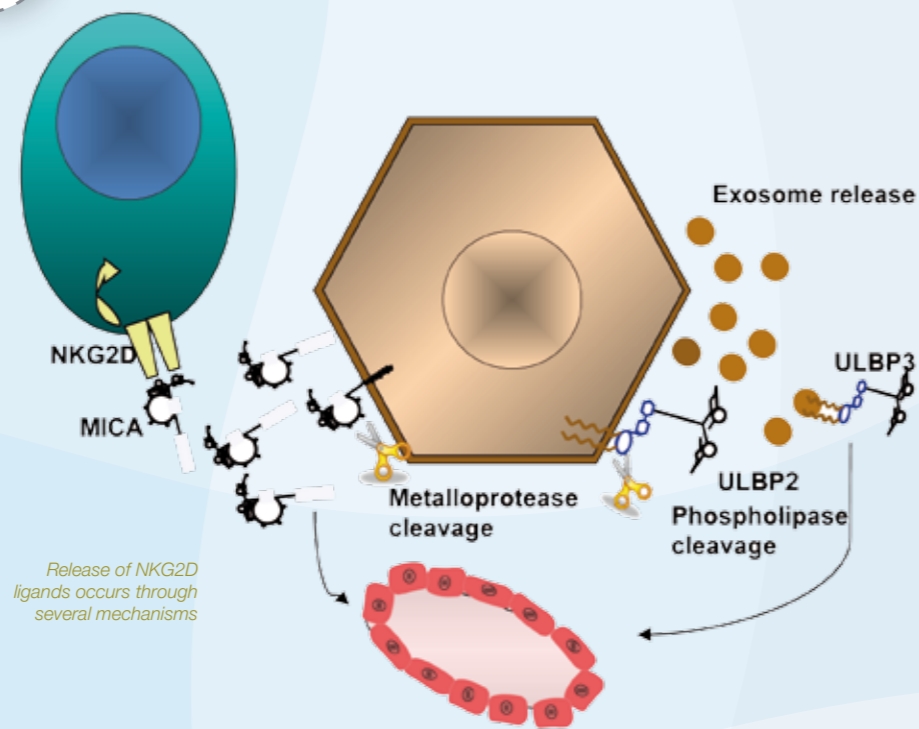
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**Biochemical
Characterization of the
Ligands for the Immune
Receptor NKG2D:
Implications of their
Heterogeneity for Pathology
and Therapy**



Release of NKG2D ligands occurs through several mechanisms

NKG2D is an activating immune receptor constitutively expressed in humans in most cytotoxic lymphocytes including NK and CD8+ T cells; in mice it is expressed on NK cells, but on T cells only after activation.

After binding of its ligands, NKG2D activates the mechanisms that lead to lysis and cytokine secretion by immune effector cells. It thus seems reasonable that NKG2D ligands are not expressed constitutively on all cell types. Instead, their pattern of expression is affected by cell stress. In

humans, NKG2D ligands (NKG2D-L) belong to two families of "stress-inducible" proteins: the polymorphic family of MHC-I related chain A/B (MICA/B) and the multi-gene family of UL16-binding proteins (ULBP, now termed RAET1A-E). The existence of such a large number of ligands for a single receptor is not fully understood, but might reflect a differential role for distinct ligands in immune surveillance or an evolutionary response to selective pressures exerted by pathogens or cancer. Our hypothesis is that the different biochemical properties of NKG2D ligands could lead them to follow distinct cellular pathways, and that these biological features would allow the cell to adapt to a variety of stress stimuli (pathogen, tumour transformation).

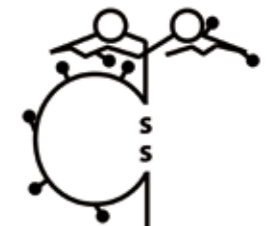
Interestingly, NKG2D ligands can also be shed as soluble molecules and induce a state of unresponsiveness in T and NK cells. This phenomenon is of particular importance in immune recognition of cancer, since the presence of soluble ligands for NKG2D in serum of cancer patients has been linked to poor disease prognosis.

Recent work from the laboratory, initiated at the University of Cambridge, focuses on studying the cellular and molecular differences that would explain the heterogeneity among NKG2D ligands. Our data demonstrate that release of NKG2D ligands occurs through several cellular mechanisms, including metalloprotease cleavage and release in exosomes.

Tumour release of proteins is a vehicle of communication with the immune system that can lead either to activation of the response and elimination of the tumour, or to suppression and immune escape. In the case of NKG2D ligands, the presence of soluble protein leads to inhibition of NK cell cytotoxicity, and exosomes containing NKG2D ligands are potent downmodulators of the NKG2D receptor.

MHC Class I-related protein (MIC)

MICA, MICB
Polymorphic



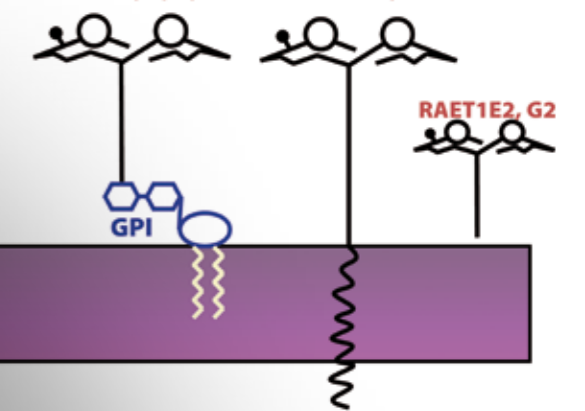
NKG2D ligands belong to two families of stress-related proteins

**UL16-Binding Proteins (ULBPs)
Retinoic acid early transcript (RAET1)**

ULBP-1, 2, 3 and 6
RAET1 I, H, N, L

ULBP-4, 5
RAET1E, G

RAET1E2, G2



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Cellular & Molecular Biology

INÉS M. ANTÓN GUTIÉRREZ
 Molecular Bases of Cytoskeletal Reorganisation: Role of Actin Polymerization in Neuritogenesis, Inflammation and Metastasis 46

LUIS ENJUANES
 Replication, Virus-Host Interactions, and Protection in Coronavirus 48

MARIANO ESTEBAN
 Poxvirus and Vaccines Lab 50

ALBERTO FRAILE
 Pathogenesis and Cell Biology of Human Cytomegalovirus 52

PABLO GASTAMINZA LANDART
 Cellular Factors Involved in Hepatitis C Virus Infection and Pathogenesis 54

FRANCISCO J. IBORRA
 Control of Cell Differentiation 56

LLUÍS MONTOLIU JOSÉ
 Animal Models by Genetic Manipulation 58

JOSÉ R. NARANJO & BRITT MELLSTRÖM
 Functional Analysis of Transcriptional Repressor DREAM 60

AMELIA NIETO
 Mechanisms of Interaction Between the Influenza Virus and the Infected Cell 62

MARTA NIETO LÓPEZ
 Cerebral Cortical Development 64

JUAN ORTÍN MONTÓN
 Transcription and Replication of Influenza Virus RNA 66

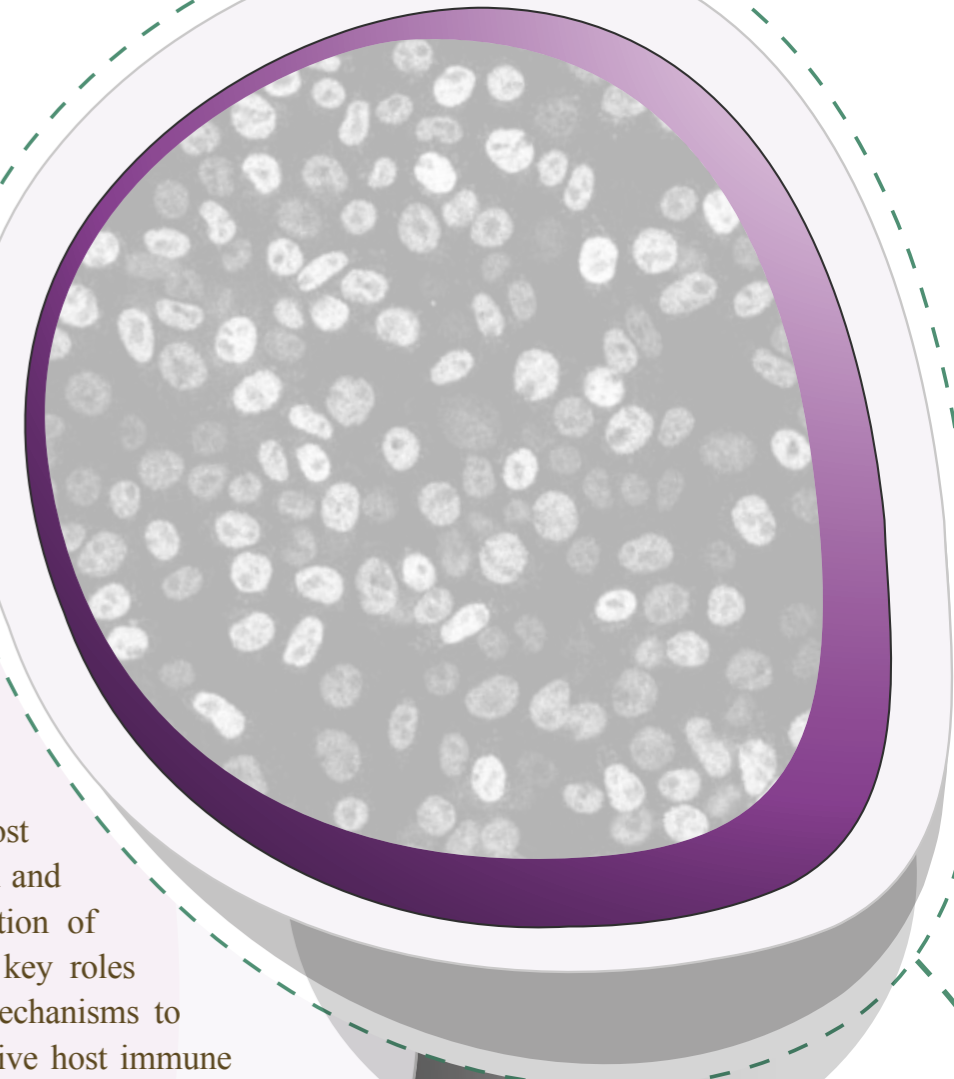
MARÍA DEL CARMEN RIVAS VÁZQUEZ
 Virus and Cancer 68

DOLORES RODRÍGUEZ AGUIRRE
 Molecular Characterization and Epidemiology of Torovirus 70

JOSÉ FRANCISCO RODRÍGUEZ AGUIRRE
 Biología Molecular de Birnavirus 72

ESTEBAN VEIGA CHACÓN
 Cellular Immunobiology and Microbiology 74

The Department hosts fifteen independent research groups working on two broad and highly intertwined research areas with the ultimate scientific goal of identifying specific therapeutic targets potentially useful for disease prevention and control. The first research area is focussed to the dissection of virus-host interactions of highly relevant human and veterinary pathogens. The identification of virus and cellular elements playing key roles in virus replication as well as on mechanisms to evade or counteract innate and adaptive host immune responses at cellular and organism level is essential for the rational design and implementation of new strategies for disease control, including the generation of innovative vaccination strategies and virus-based vaccine vectors. The second area is centred on the understanding of networks controlling mammalian gene expression, and on the characterization specific genes playing critical roles on both normal and pathological processes. The focal point of this research program is the identification and exploitation of relevant diagnostic and therapeutic molecular targets. In addition to the generation of frontier knowledge, studies carried out at the Department are intended to provide the essential scientific background for the development of novel biotechnological tools of biomedical importance.



Cellular & Molecular Biology

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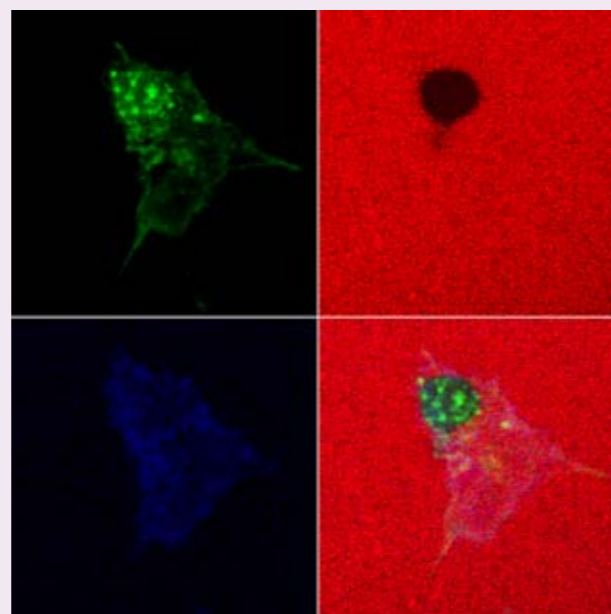
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Molecular Bases of Cytoskeletal Reorganisation: Role of Actin Polymerization in Neuritogenesis, Inflammation and Metastasis

Essential physiological processes such as neuronal morphogenesis, cell motility and tissue invasion rely on the spatial and temporal regulation of actin dynamics and therefore, their deregulation is at the root of severe pathologies.

Actin reorganization is controlled by nucleation-promoting factors like neural Wiskott-Aldrich syndrome protein (N-WASP) and cortactin, and associated proteins that regulate their activity such as WIP (WASP-interacting protein), a ubiquitously distributed protein that stabilizes actin filaments. Our goal is to define the role of WIP- and WASP-family proteins in actin dynamics within a variety of cellular processes in an array of cell models (fibroblasts, dendritic cells (DC), neurons and astrocytes).



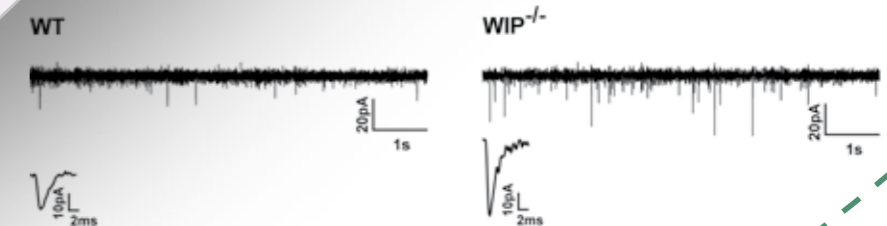
WIP expression restores podosome formation and ECM degradation. WIP KO DC infected with a lentivirus coding for WIP-eGFP (green) were plated onto gelatin-rhodamine (red) and labelled with anti-cortactin (blue).

In the past two years, we have contributed toward defining the molecular mechanisms that underlie invasion and extracellular matrix (ECM) degradation by matrix metalloproteinases (MMP). We described that WIP is essential for MMP secretion, but not for MMP synthesis, in murine DC. Moreover, absence of WIP prevents the formation of podosomes, actin-rich migratory structures in which MMP activity localizes. Cortactin binding to WIP is required for correct podosome formation, MMP activity and ECM degradation. Our future work aims at unravelling the WIP contribution to invasion and metastasis by human tumour cell lines.

Since the ability to reorganize actin filaments is at the basis of neuronal plasticity, we studied the role of WIP on neuron differentiation both in early and in late developmental stages, *in vitro* as well as *in vivo*. Using embryonic hippocampal primary WIP^{-/-} neurons, we characterized, at the biochemical and morphological levels, the effect of WIP as a negative regulator of sprouting and neuritic/dendritic branching without affecting axon generation. Similar to our observations in dendritic cells, we found that WIP expression in neurons is required for the correct cortactin localization. Our results point to WIP as a novel regulator that prevents premature dendritic and synaptic maturation. We aim to deepen our understanding of the contribution of WIP to neuron and astrocyte migration, to brain lamination and development, and the functional effects of WIP deficiency on the murine nervous system.

The results of our work should yield fundamental information on the function of these cytoskeletal molecules and offer new insights into the molecular mechanisms that underlie actin dynamics and related functions, providing new diagnostic and/or therapeutic tools for neurological diseases, inflammation-mediated affections and tumour invasion.

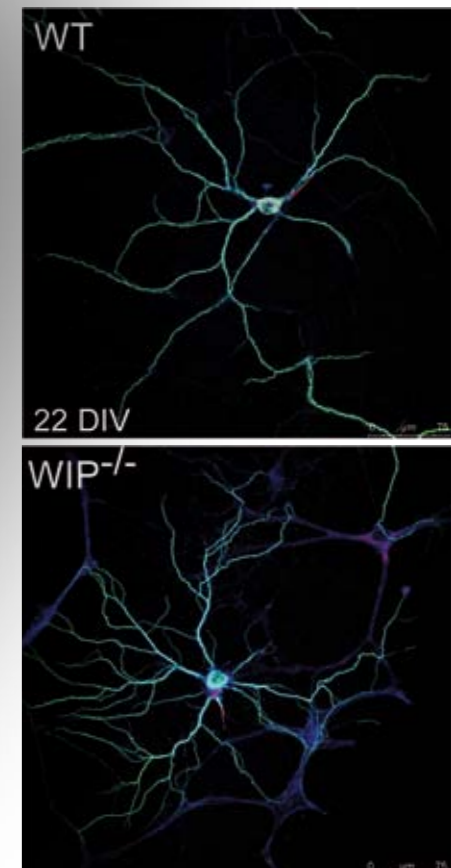
Enhanced dendritic and synaptic maturation in WIP^{-/-} neurons. Top, Hippocampal primary neurons from control (WT) or WIP^{-/-} embryos were grown for 22 days (DIV) onto an astrocyte monolayer and stained for F-actin (blue), MAP2 (green) and ankyrin G (red). Bottom, Representative traces of miniature excitatory postsynaptic currents recorded from a WT and a WIP^{-/-} neuron. Amplitude (pA) and frequency (s) are indicated.



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Cellular & Molecular Biology

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Replication, Virus-Host Interactions, and Protection in Coronavirus

The main focus of our research is the study of the molecular basis of coronavirus (CoV) replication and virulence, and the identification of signalling pathways modified by the virus, to control disease.

The information from these basic projects will be used to design protection strategies against CoV-induced diseases, particularly human severe pneumonia that can end in acute respiratory distress syndrome (ARDS).

The CoVs are single-stranded positive-sense RNA viruses with genomes of around 30 kb, responsible for respiratory and enteric diseases with high impact on animal and human health. Our group is interested in the molecular basis of replication and transcription, assembly, and virus-host interaction using transmissible gastroenteritis coronavirus (TGEV) and the severe and acute respiratory syndrome virus (SARS-CoV) as models.

Virus replication and transcription, and virus-host interactions are mediated by binding of virus RNA motifs to viral and host cell proteins, and by protein-to-protein recognition. These interactions are analysed in all the processes that we study, including the replication complex. We postulated that coronavirus transcription and replication involve 5' and 3' genome end interactions, mediated by proteins that are being identified using functional genomics and proteomics. The relevance of host cell factors involved in these processes is being evaluated by inhibiting their expression using siRNAs.

Coronavirus transcription requires discontinuous RNA synthesis to link the leader to coding sequences in the subgenomic RNA, a process similar to high frequency copy-choice, similarity-assisted RNA recombination. Based on a large amount of data generated by reverse genetics, we described two mechanisms that regulate transcription in coronaviruses at different levels. We showed that basepairing between the nascent minus RNA chain and the genomic RNA leader regulates the amount of all subgenomic RNAs produced. A transcription enhancer that regulates the expression of a specific mRNA was also recently identified. There are ad-

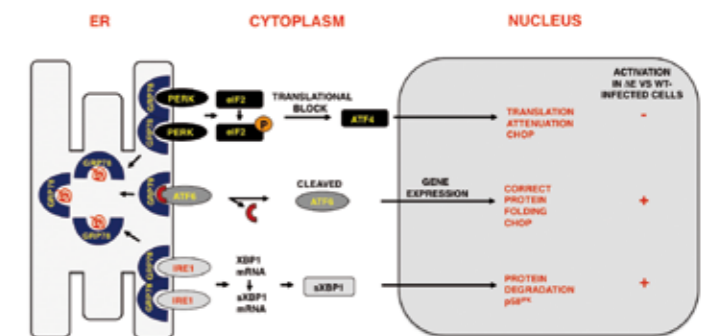
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UNFOLDED PROTEIN RESPONSE



SARS-CoV gene E and unfolded protein response. A scheme of the three pathways (PERK, ATF-6 and IRE1) comprising the unfolded protein response (UPR). Infection with a SARS-CoV deletion mutant lacking gene E expression (ΔE) enhanced UPR compared with wt virus infection. This effect was due mostly to the activation of IRE1 pathway, leading to increased XBP1 cleavage.

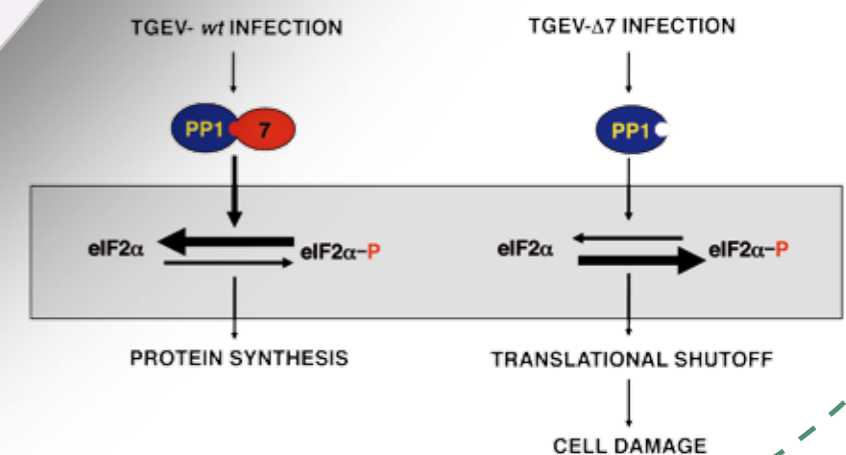
Role of coronavirus alpha gene 7 on translation. After TGEV infection, viral protein 7 interacts with protein phosphatase 1 (PP1). This interaction promotes eIF2 α dephosphorylation, leading to protein translation. Infection with a TGEV mutant lacking gene 7 expression ($\Delta 7$) increased eIF2 α phosphorylation, leading to translational shutoff and enhanced apoptosis.

ditional regulatory mechanisms that influence the amount of subgenomic RNA, such as RNA-protein interactions, one area in which our laboratory is highly active at present. The role of viral and cell proteins, such as RNA chaperones, that might be involved in CoV replication, transcription and packaging is being addressed; we showed that coronavirus N protein is essential for coronavirus RNA synthesis and acts as an RNA chaperone.

A main area of interest in our laboratory is the study of the molecular basis of virus virulence, virus-host interaction, and signalling pathways affecting virus replication or, alternatively, cell pathways altered by coronavirus infection leading to diseases such as those associated with inflammation of respiratory tissues. Comparative genomics and proteomics information is essential in these studies. We described that specific virus structural proteins, such as the TGEV and SARS-CoV envelope (E) proteins, influence virus virulence and modulate signalling pathways. The deletion of E protein led to the generation of propagation-deficient TGEV, to attenuated phenotypes in the case of SARS-CoV, and to upregulation of the cell stress response, which affects the immune response. Deletion of non-essential virus components such as TGEV protein 7 significantly affects viral and cell translation and apoptosis. Viral and host cell factors involved in these signalling pathways are being studied.

The information derived from academic studies is being applied to the engineering of coronavirus vectors. Using reverse genetic approaches based on two infectious cDNA clones produced in our laboratory for TGEV and SARS-CoV, viral vectors have been engineered using virus-attenuated phenotypes. These vectors have led to promising recombinant vaccine candidates for animal and human health, including SARS.

WORKING HYPOTHESIS



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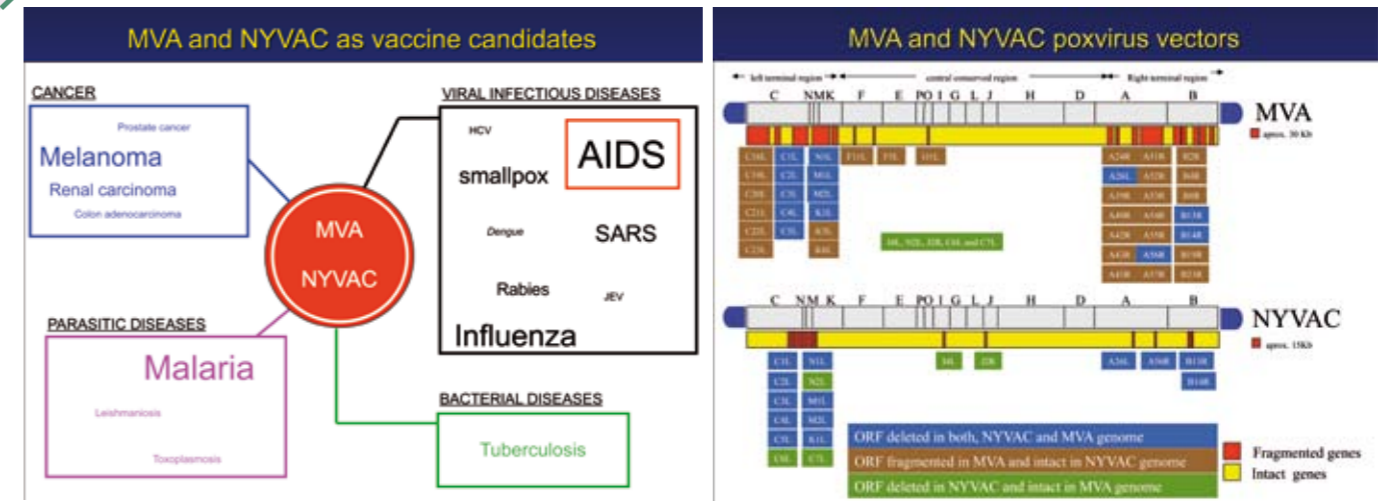
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Poxvirus and Vaccines Lab

The main objectives of our laboratory are geared toward understanding the molecular basis of the pathogenesis of infectious agents and their interaction with the host, as well as to use this knowledge in the development of vaccines that might be effective against diseases like AIDS, malaria and leishmaniasis.

As a model system of an infectious agent and as a delivery vector for expression of genes of interest, we used vaccinia virus (VV), a member of the poxvirus family.

Our group at the CNB has developed prototype poxvirus (NYVAC and MVA strains) vaccines against HIV/AIDS based on subtypes B and C, which account for nearly 80% of all HIV infections worldwide. In preclinical studies in mice and in macaques, the prototype vaccines expressing four HIV antigens (Env/Gag-Pol-Nef) have fulfilled the expected characteristics of a potentially effective vaccine, i.e., high immunogenicity; in monkeys, they elicit protection after challenge with pathogenic simian immunodeficiency virus; the vaccines can be delivered safely by aerosol, which facilitates their administration, especially in poor countries; and when given to human healthy volunteers in DNA/poxvirus combination, triggered HIV-specific T cell immune responses in over 90% of volunteers, with a polyfunctional and durable immune response. With the vaccine prototype MVA-B, in 2009 we initiated a phase I clinical study in Spain, with the participation of the Hospital Clinic in Barcelona and the Hospital Gregorio Marañón in Madrid, which has received wide attention from the media. Follow-up phase I clinical trials in Spain will be started in 2011, with only MVA-B administered in HIV-positive individuals on HAART therapy, to assess safety and immunogenicity of the vaccine protocol. Phase I clinical trials with the MVA-C and NYVAC-C prototypes will be started in Africa in 2011. To determine the impact of these vaccines on human cells, we have identified gene signatures triggered by the HIV/AIDS vaccine MVA-B that might play important roles in the innate immune response. We also identified the TLR trig-



Attenuated poxvirus vectors as vaccine candidates against prevalent human diseases and, diagram showing genome deletions in the two viral vaccine candidate vectors MVA and NYVAC.

gering signals induced by the poxvirus vectors. To potentiate the immune responses to the poxvirus vectors, we followed two strategies: use of adjuvants such as Mega CD40L and selected deletions in viral immunomodulatory genes. In collaboration with other groups, we have provided important insights into the structural organization of the virus core and the role of some oncogenes in antiviral action.

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Cellular & Molecular Biology

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Pathogenesis and Cell Biology of Human Cytomegalovirus

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is responsible for a number of diseases in immunocompromised patients.

Furthermore, HCMV infection has been associated with colon cancer and gliomas, but there is no evidence supporting a role for HCMV-encoded genes in cancer pathogenesis. As HCMV is an enveloped virus, replication is dependent on interactions with cell membrane systems. The mechanisms of HCMV envelopment and secretion nonetheless remain to be defined. In our group, we study molecular mechanisms that underlie HCMV pathogenesis and morphogenesis. To investigate the molecular basis of pathogenesis, we study the role of the HCMV-encoded chemokine receptor US28 in cancer. To provide new insights into viral morphogenesis, we explored the function of cell components involved in vesicle-mediated transport, including small GTPases of the Rab family and SNARE proteins, in this process.

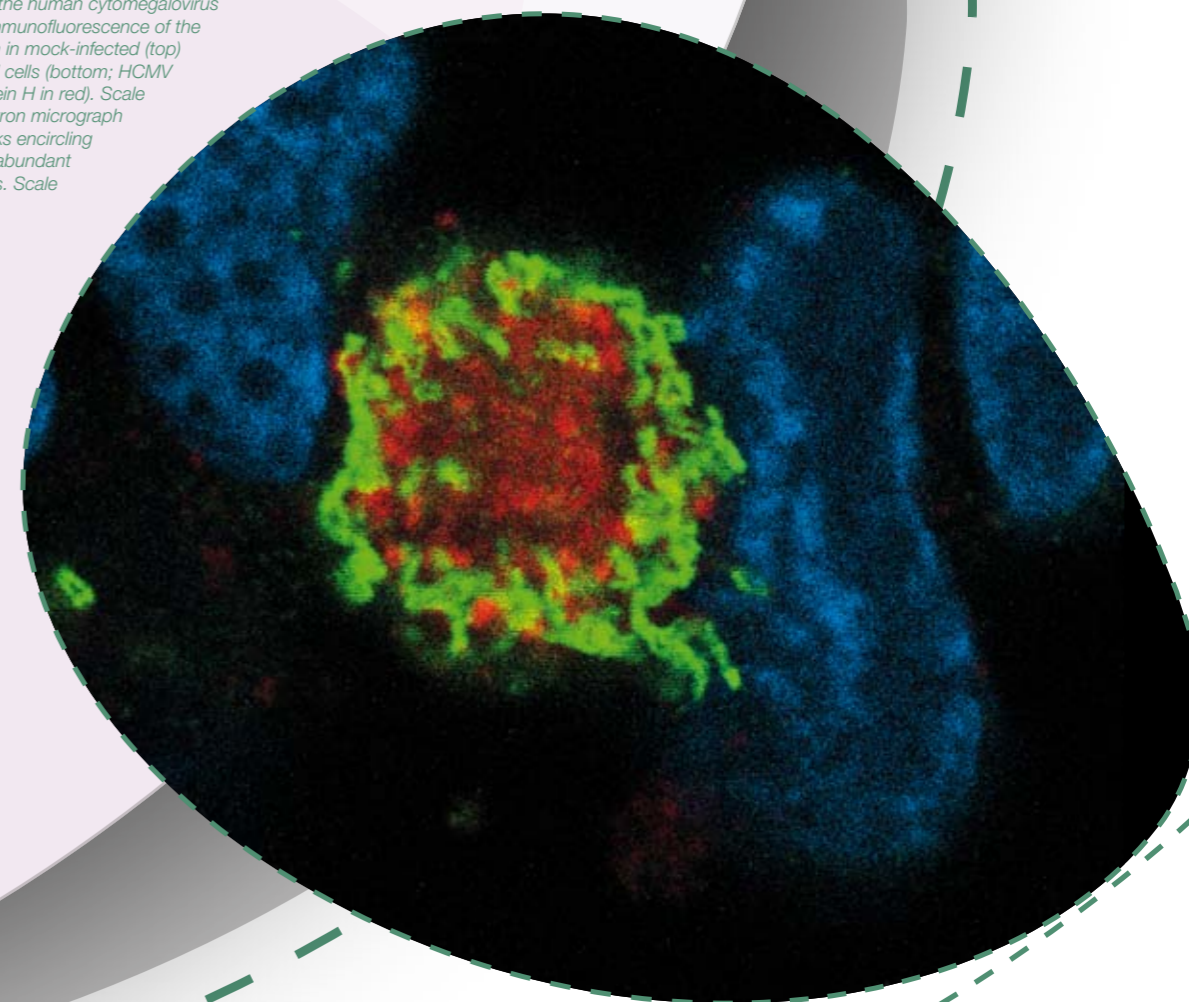
We found that US28 expression in transgenic mice promotes development of dysplasia. We also showed that US28 proliferative signalling occurs through the interleukin-6/signal transducer and activator of transcription 3 (STAT3) axis. Moreover, analyses of tumour specimens from glioblastoma patients demonstrated colocalisation of US28 and phosphorylated STAT3 in the tumours.

We observed that HCMV induced the expression of Rab27a, a small GTPase involved in lysosome-related organelle transport, as well as its recruitment to the assembly site where Rab27a associated with viral envelopes. CMV production was reduced after silencing of Rab27a and in Rab27a-deficient ashen melanocytes. This study indicates a role for Rab27a in the CMV life cycle, and that CMV and LRO biogenesis share common molecular mechanisms. In addition, we found that HCMV induces expression of the SNARE protein syntaxin 3 (STX3). STX3 was located at the plasma membrane and at the assembly site, where it is associated with virus wrapping membranes. STX3 silencing

reduced HCMV production; in contrast, STX3 depletion did not affect herpes simplex virus type-1 production. The mechanism through which STX3 affected HCMV morphogenesis probably involves late endosomes/lysosomes, since STX3 depletion reduced expression of lysosomal membrane glycoproteins. These results demonstrate a function for STX3 in HCMV morphogenesis, and help unravel the role of this SNARE protein in late endosome/lysosome compartments.

In summary, our work provides new insights into the basis of HCMV pathogenesis and replication and also shed light on basic aspects of cell membrane biology. The results of our work might suggest novel targets for antiviral strategies against these important pathogens.

Characterisation of the human cytomegalovirus assembly site. A) Immunofluorescence of the Golgi marker giantin in mock-infected (top) and HCMV-infected cells (bottom; HCMV envelope glycoprotein H in red). Scale bar, 20 µm. B) Electron micrograph showing Golgi stacks encircling virus factories with abundant vesicles and tubules. Scale bar, 250 nm.



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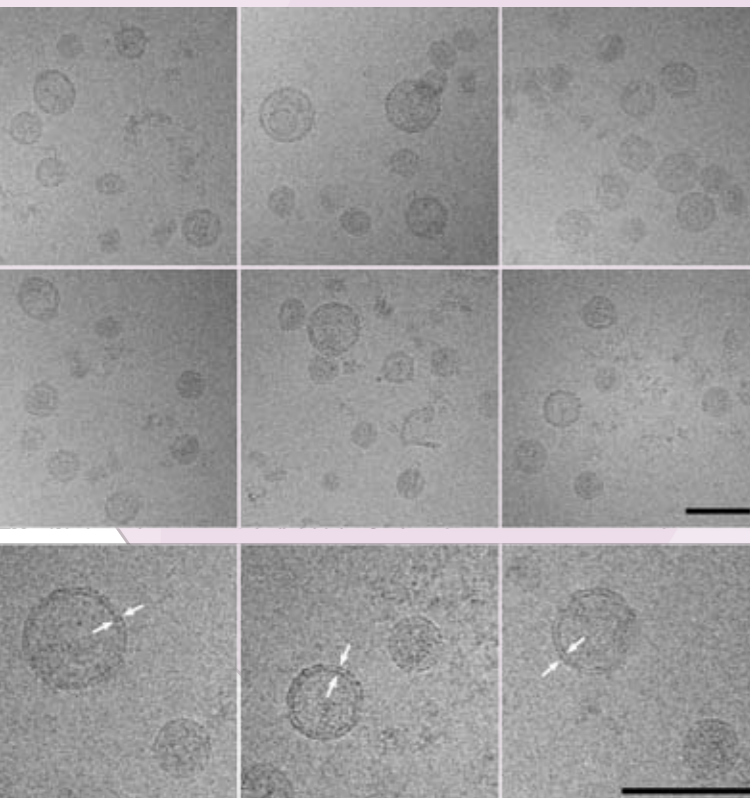
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Cellular & Molecular Biology

LEAD INVESTIGATOR

Pablo Gastaminza Landart



Cellular Factors Involved in Hepatitis C Virus Infection and Pathogenesis

Hepatitis C virus (HCV) is a pathogen that infects 3% of the human population worldwide.

Our laboratory is interested in the cellular and molecular processes underlying aspects of HCV biology and pathogenesis. After contributing to the development of a cell culture model for HCV infection, we focused on the process of infectious virus assembly and virion morphology. Previous studies at the Scripps Research Institute (La Jolla, CA) showed that infectious HCV assembly relies on cellular lipoprotein biosynthesis, and that lipoprotein components are incorporated into HCV particles. We showed that apolipoprotein B (apoB) expression levels are rate-limiting for HCV assembly and that apolipoprotein E (apoE) is incorporated into HCV particles. To determine how viral and cell components are organised in the infectious virions, we optimised virus production and purification, and obtained virus preparations that enable the study of virion morphology by cryoelectron microscopy. This analysis revealed that HCV particles are pleomorphic and heterogeneous in size, some of which (those associated with greatest infectivity) show a visible envelope surrounding an apparently disordered capsid. At the CNB, we are conducting biochemical and functional studies to determine the molecular mechanisms that underlie the dependence of HCV assembly on apoB, apoE and other cellular genes involved in lipid metabolism, and how this relationship might alter normal cell lipid homeostasis.

In addition to studying cellular factors that mediate HCV infection, we explored new methods to identify molecules with antiviral potential against HCV. We developed a novel unbiased cell-based screening system that enables interrogation of chemical libraries for compounds that target

CryoEM micrographs of highly purified HCV particles showing heterogeneous size and morphology of the viral population. Some particles display a visible bilayer that probably represents the viral envelope (white arrows).

known and unknown aspects of the viral life cycle, be they cellular or viral. We established the efficacy of this system using a library of clinically approved compounds and demonstrating the capacity of the system to identify compounds that inhibit entry, replication and assembly processes, as well as compounds that target undefined aspects of HCV infection. Not surprisingly, some of the antiviral compounds target cellular factors involved in lipid metabolism regulation. After this proof of principle, we interrogated a chemical library of compounds susceptible to chemical derivatisation using click chemistry, a highly modular and predictive synthetic method, optimal for structure-activity relationship studies. The screening process led to identification of a new family of anti-HCV compounds, and their derivatisation permitted us to optimise the antiviral molecules to obtain compounds with antiviral activity at nanomolar concentrations. Study of the mechanism of action showed an unprecedented mode of action at the outset of infection, in which the non-structural protein NS5A is involved after primary translation and leads to the establishment of replication complexes.

Micrograph of a HCV infection focus, determined by immunofluorescence microscopy using antibodies against viral antigens (red). Cells are counterstained with DAPI. Counting infection foci permits calculation of infectivity titres (focus-forming units per ml; FFU/ml) in samples from infected cells.

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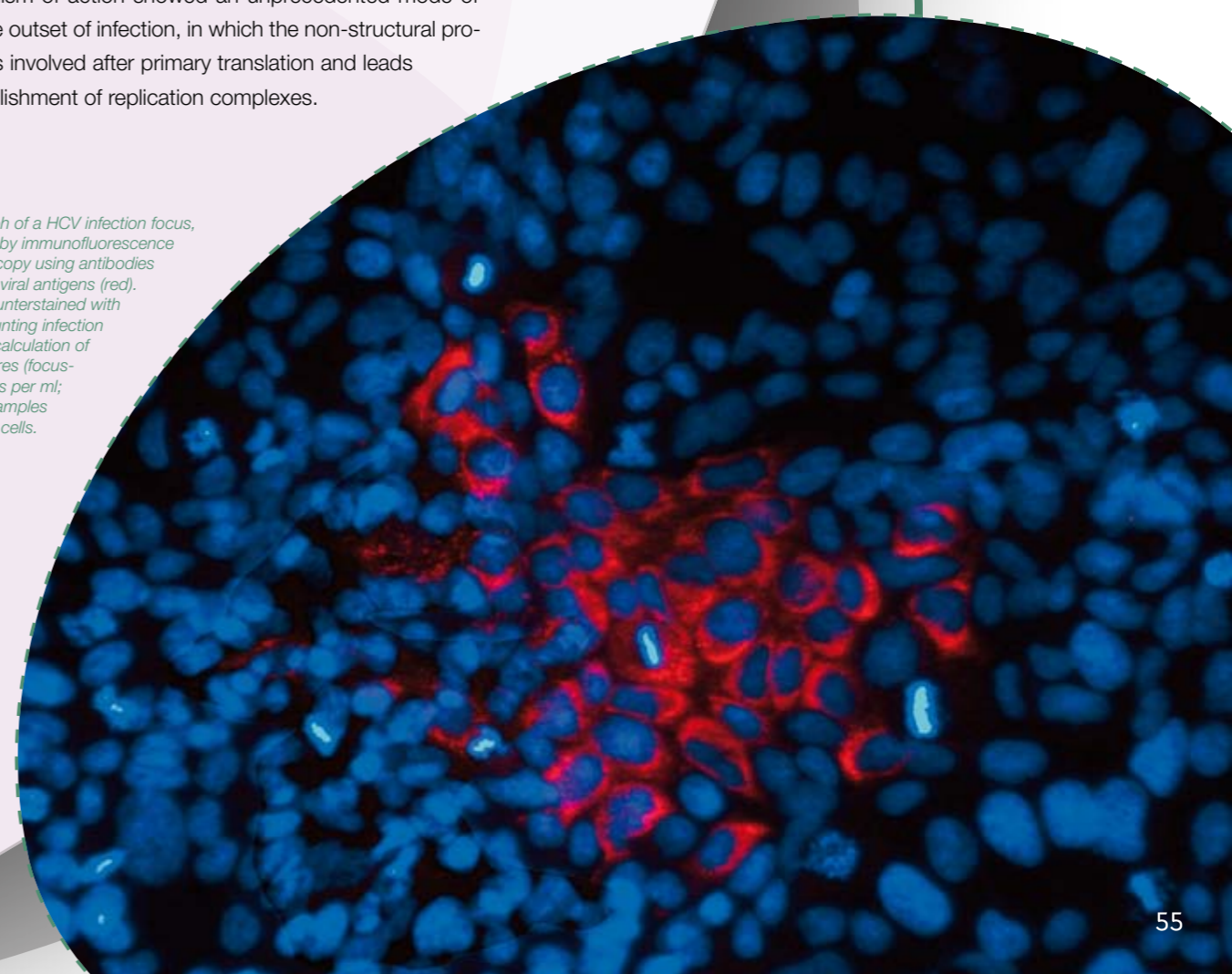
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Cellular & Molecular Biology

LEAD INVESTIGATOR

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Control of Cell Differentiation

We would like to understand the fundamental question of the control of cell differentiation, how a cell moves from one genetic program to another.

The difference between the cells of our body is the set of genes they express. Pathological situations tend to be associated with aberrant patterns of gene expression. Gene expression starts at the DNA, which can be modified to be silent. The second control level is the kind of modification of the proteins wrapping the DNA, again rendering the gene silent or active. Third, the right protein factors must direct the transcription machinery to the site at which to start transcribing the gene. These proteins work in teams, and the relative abundance of each will drive the cell to take one path or another. Our group plans a detailed analysis of these factors at the single cell level, to understand the regulation of cell differentiation and pathology.

All cells in the organism are derived from the same progenitors through differentiation, which can be seen as a progressive change in phenotype. The phenotype of any cell is ultimately determined by the set of genes transcribed, which are determined by specific transcription factors and epigenetic modifications of chromatin in the cell nucleus.

This idea was recently challenged by the observation that genetically identical populations of cells can exhibit cell-to-cell variations in the amount of protein a gene produces, resulting in phenotypic diversity. It is thought that the variations arise from the typically small number of molecules

involved in gene expression. Protein numbers are often in the order of hundreds of molecules, mRNA in the order of tens of molecules, and the genes themselves are often present in just one or two copies per cell.

We attempt to understand how the organisation of transcription in the nucleus affects haematopoietic cells during differentiation, how transcription factors and extrinsic factors modulate gene expression. These analyses will provide some essential rules to understand how gene expression is integrated in the nuclear context and how nuclear structure and cellular processes influence gene activity.

The factors leading to cell-to-cell variation can be classified as originating from two sources: (a) variations in global, or extrinsic, factors, such as varying amounts of transcriptional activators, or (b) essentially random, or intrinsic, molecular events, such as the transcription of a particular gene that is regulated by a specific set of activators.

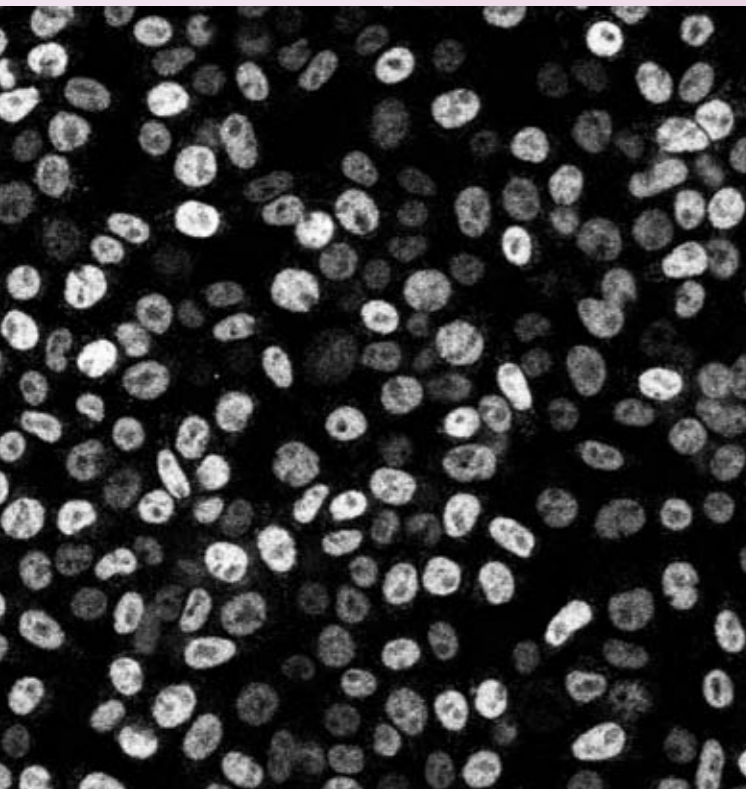
My group studies the contribution of the different factors to cell-to-cell variation, using single-cell and genomic approaches. Our long-term goal is to build a detailed map of processes such as cell differentiation and transformation.

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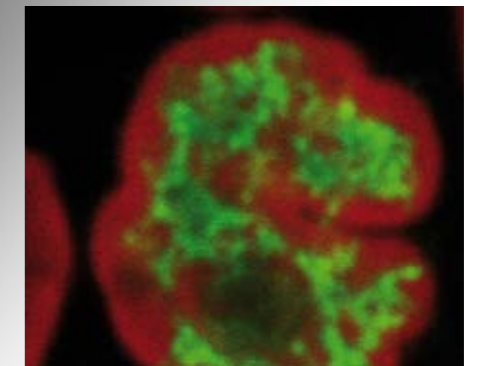
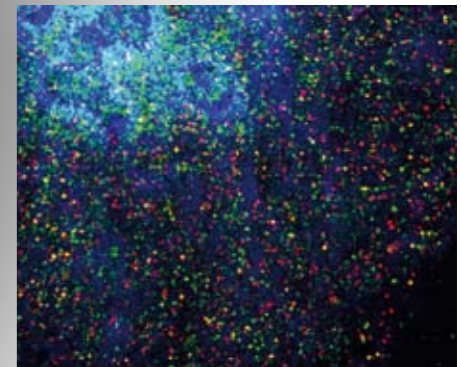
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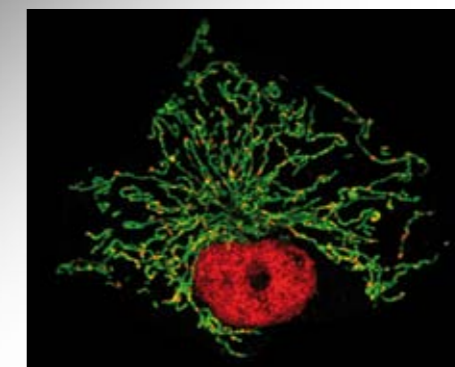
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Noise in transcription.



HeLa cells grown in presence of BrU, as a precursor of transcription, show a high degree of variability in Br-RNA production.



Cellular & Molecular Biology

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Animal Models by Genetic Manipulation

In our laboratory, we are interested in understanding how mammalian expression domains work and how they are organised within genomes.

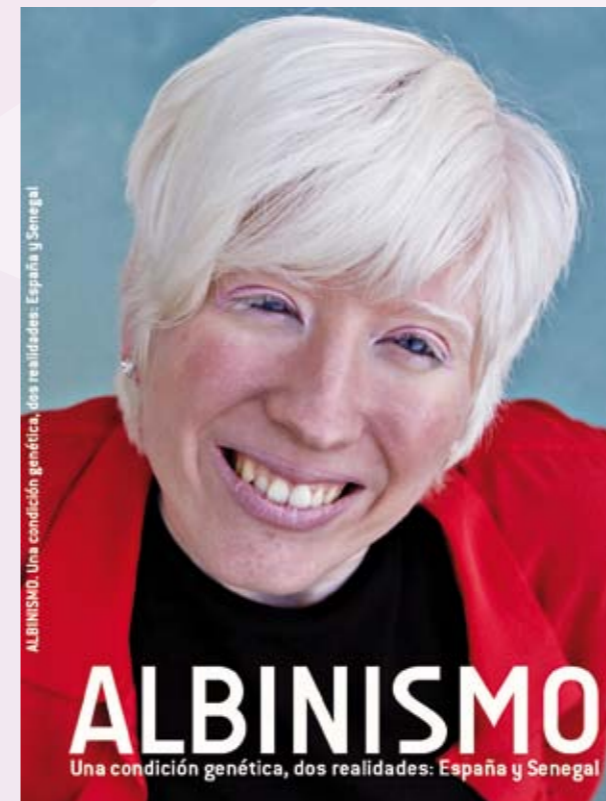
In particular, we focus on the identification and characterisation of genomic boundaries or insulators. By studying insulator elements, we aim to contribute to understanding of the functional and structural organization of vertebrate genomes. Insulators can be used effectively in biotechnological applications, as spacers, as boundaries, in any gene expression construct to be used in gene transfer experiments. They prevent inappropriate expression transgenes or gene therapy constructs and insulate them from neighbouring sequences at the insertion site in the host genomes. We are searching for new insulator sequences in vertebrate genomes, through initial bioinformatic analyses. Insulator candidates are functionally validated *in vitro* using cells and the enhancer blocking assay, and possibly *in vivo*, using transgenic animals bearing appropriate constructs (zebrafish in collaboration with JL Gómez-Skarmeta at the CABD, and mice at the CNB).

In addition, our laboratory generates and analyses new animal models to study neural alterations in vision, as well as in hearing, associated with albinism, a rare disease whose research effort is the focus of work within the CIBERER (www.ciberer.es). Using transgenic pigmented and albino mice in collaboration with the laboratory of I Varela-Nieto (IIB-CSIC/UAM), we showed that albino mice display premature severe hearing loss and do not recover after a noise-induced hearing loss, compared to their pigmented counterparts.

We collaborate with ALBA (www.albinismo.es), the Spanish association in support of people with albinism, and have published a book to inform society about the different types of albinism and their phenotypic consequences.

The expertise of our laboratory and its leadership in the Mouse Embryo Cryopreservation and Histology Facilities at the CNB have also been fundamental for our participation in the FP7 European Projects within the field of mouse functional genomics (INFRAFRONTIER) and in EMMA,

the European Mouse Mutant Archive, whose Spanish node at the CNB began operation in 2009 under our coordination. This work has produced additional publications within the field of animal transgenesis.



Cover of the book on albinism published by ALBA (2009) with the collaboration of L. Montoliu (photo by A Yturralde).

Finally, through collaborations, we have generated and are analysing a number of additional animal models (transgenic mice) to study human diseases, including Alzheimer, exploiting our technology of yeast artificial chromosome (YAC)-type of transgenes, which have been instrumental in the execution of scientific contracts with biotechnology and pharmaceutical companies.

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Transgenic zebrafish expressing GFP, used to validate insulator function of selected vertebrate genome elements. See Bessa et al. (2009) for additional information (photo by JL Gómez Skarmeta (CABD)).

Cellular & Molecular Biology

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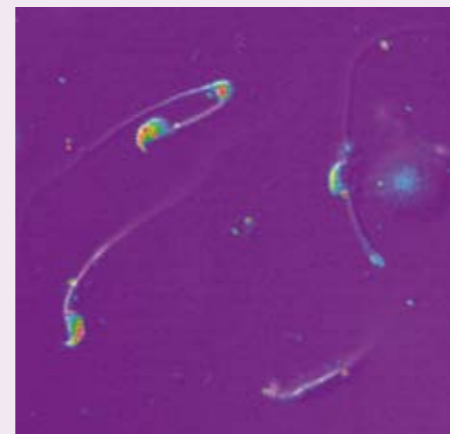
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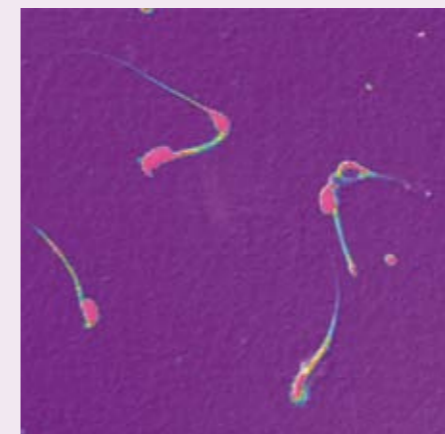
Functional Analysis of Transcriptional Repressor DREAM

We study the nuclear components of activity- and Ca²⁺-dependent transcriptional responses in neurons and immune cells to

i) understand the molecular determinants of downstream events responsible for plastic changes in synaptic function, ii) to regulate the output of physiological functions including learning and memory, pain sensitization and immune response, and iii) to develop tools to intervene in pathological processes during neurodegeneration. We foresee the Ca²⁺-dependent transcriptional repressor DREAM as an active/central component of several nucleoprotein complexes that mediate specifically the various transcriptional cascades triggered



DREAM regulates Ca²⁺ mobilization in sperm cells. The calcium signal (red) in response to progesterone (top) is reduced in transgenic spermatozoa overexpressing DREAM (down).



DREAM regulates thyroid gland development. High DREAM levels result in abnormal growth of the thyroid.

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Cellular & Molecular Biology

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Mechanisms of Interaction Between the Influenza Virus and the Infected Cell

Influenza virus employs an unusual RNA transcription mechanism that uses as primers short-capped oligonucleotides scavenged from newly synthesised RNA polymerase II (RNAP II) transcripts; this fact entirely decides its life cycle.

It first requires functional coupling between viral and cellular transcription machineries. Second, viral and cell mRNAs contain a 5' cap structure and a 3' polyA tail and are thus structurally equivalent; influenza virus must therefore have developed sophisticated strategies to discriminate and favour translation of its own mRNA. As a consequence of its transcription mechanism and the nature of the viral mRNA, influenza virus thus requires a complex system of virus-host factor interactions to complete a successful viral replication cycle. The viral polymerase plays a key role establishing productive interactions with host-cell factors involved in both cell transcription and translation.

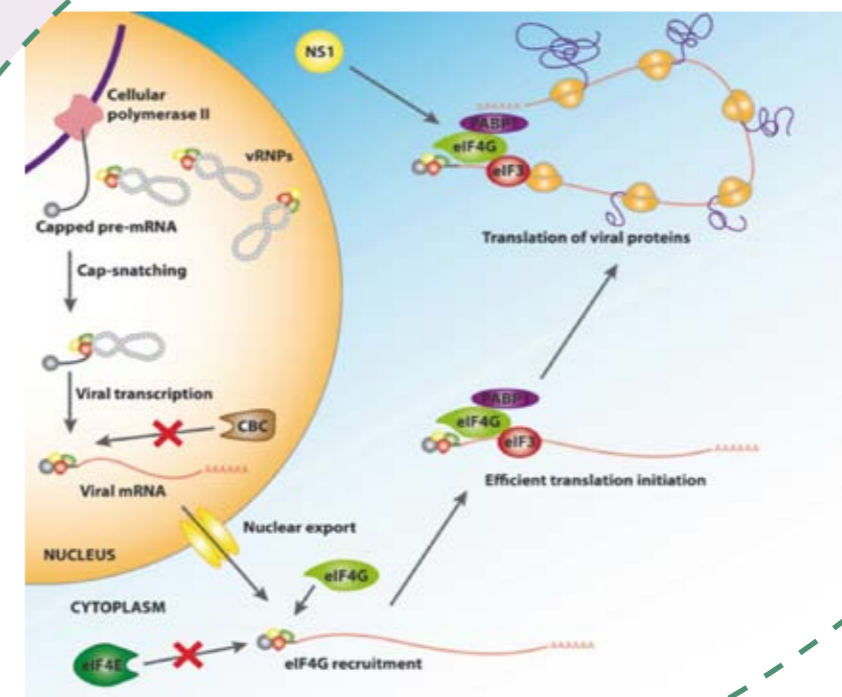
Of the cell factors that interact with viral polymerase, we have been involved in the characterization of two transcription related factors: hCLE, a positive modulator of the RNAP II and CHD6, a chromatin remodeller. We observed that whereas hCLE also positively modulates influenza virus replication, CHD6 is a negative modulator.

Although viral and cell transcription are functionally coupled, degradation of cell RNAP II occurs once synthesis of viral mRNAs is complete, probably avoiding competition; this degradation appears to be a virulence marker. Using recom-

Model for viral mRNA translation. Viral transcripts generated in the nucleus are transported to the cytoplasm with the polymerase bound. This situation avoids the replacement by eIF4E, permitting the recruitment of translation initiation complexes through the eIF4G binding capacity of the polymerase. In addition, the interaction of NS1 with eIF4G and PABP1 could help to the formation of a "closed loop" between the 5' and 3' ends of the viral mRNA activity co-operating to recruit translation complexes.

binant viruses containing polymerase segments from strains that do or do not induce RNAP II degradation, we observed that PA and PB2 polymerase subunits contribute individually to the degradation process.

Among the mechanisms that influenza virus uses to ensure selective translation of its mRNA, the eIF4F translation initiation complex has a key role. We characterized the influenza virus mRNA translation requirements for the components in this complex. Whereas eIF4A and eIF4G components are absolutely necessary, the cap-binding factor eIF4E is dispensable. Despite the fact that influenza virus mRNAs are capped, their translation is therefore independent of the cell cap-binding factor used by cellular mRNAs, and the viral polymerase might play the role of specific viral cap-binding complex for viral mRNA translation.



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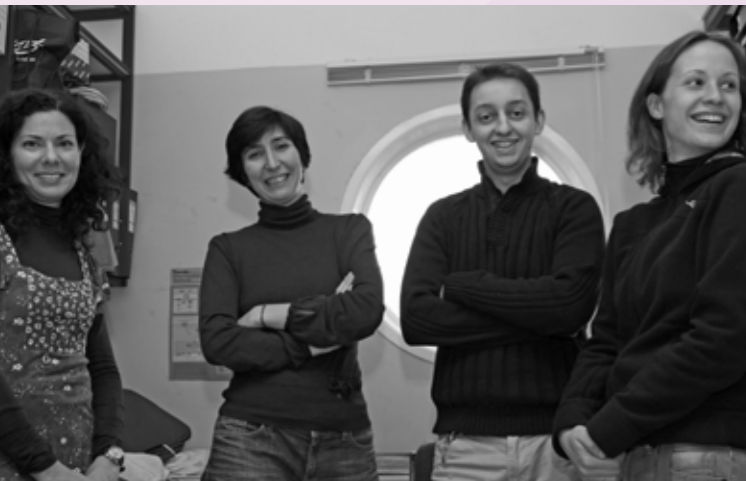
Cellular & Molecular Biology

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Cerebral Cortical Development

Human cognition is rooted in the exact formation of stereotyped complex patterns of connectivity among an enormous diversity of neurons during cerebral cortical development.

A fundamental question is to understand how the neurons of the cerebral cortex establish these precise patterns of connectivity. The cortex is organized in several functional and anatomical areas that are interconnected through stereotyped networks. Radially, the vertebrate cortex is organized into several neuronal layers (layers I-VI), and each layer contains neurons with similar molecular identities and connectivity patterns. In general, the selective expression of transcription factors (TF) in each layer and area couples the differentiation of neuronal subtypes to the establishment of their connectivity during embryonic and early postnatal stages.

Our most recent work, using knockout and knockdown studies combined with morphological, molecular and electrophysiological analysis, demonstrates that *Cux1* and *Cux2* are intrinsic and complementary regulators of dendrite branching, spine development, and the functional synapse of neurons of layer II-III of the cerebral cortex. *Cux* genes control the number and maturation of dendritic spines, partly through direct regulation of the expression of *Xlr3b* and *Xlr4b*, chromatin remodelling genes previously implicated in cognitive defects. We identified *FAM9A, B* and *C* (Martinez-Garay et al., 2002) as the closest orthologues of *Xlr* genes in humans, and found that *Cux1* and *2* proteins bind to sites in *FAM9A, B* and *C* loci that are conserved between primates and humans. This indicates that it is possible that similar *Cux*-mediated synaptic mechanisms act in humans. Downstream of *Cux*, we also found mechanisms of synaptogenesis key to cognition, including the downregulation of protein levels of NMDA receptor 2B (NMDAR2B), PSD95 and β -actin. Accordingly, abnormal dendrites and synapses in *Cux2*^{-/-} mice correlate with reduced synaptic function

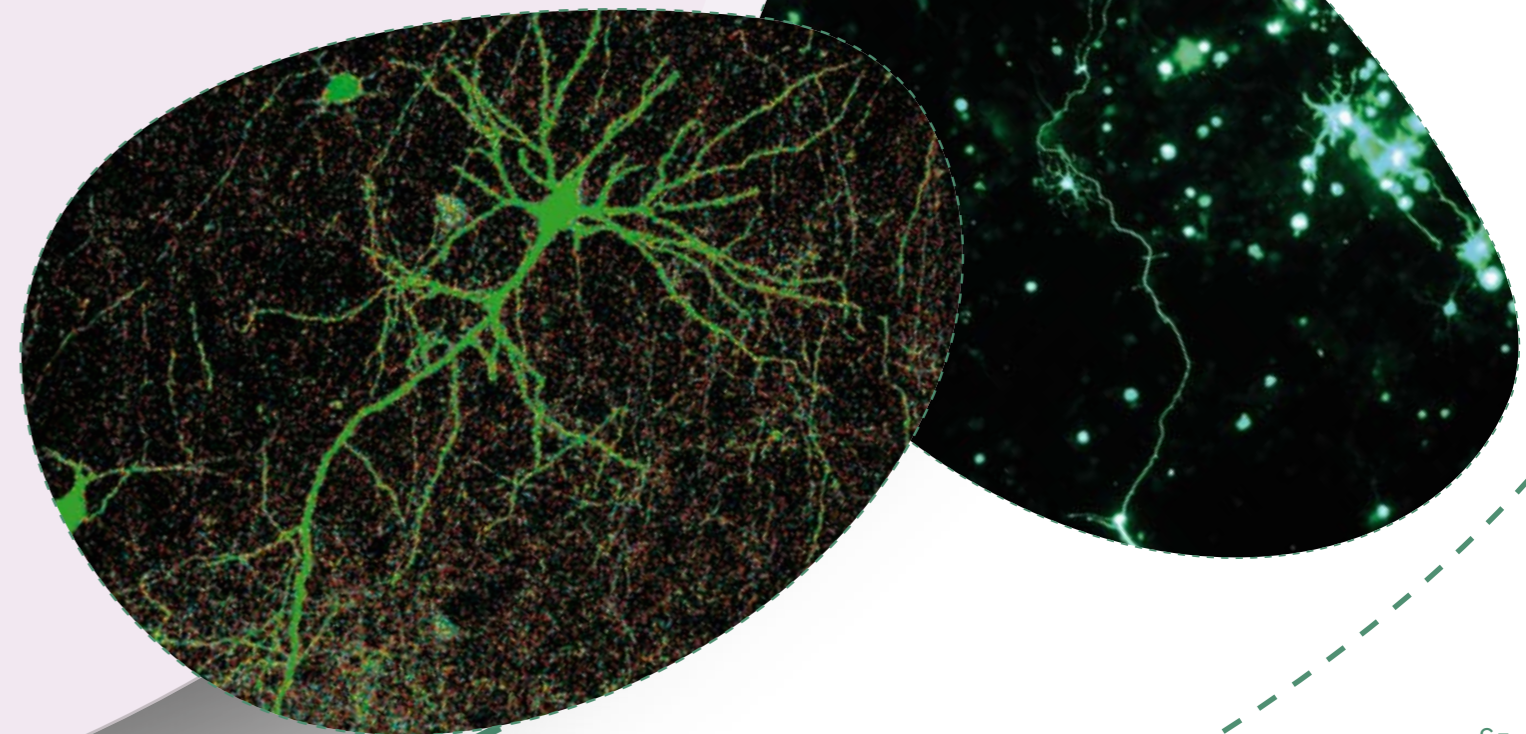
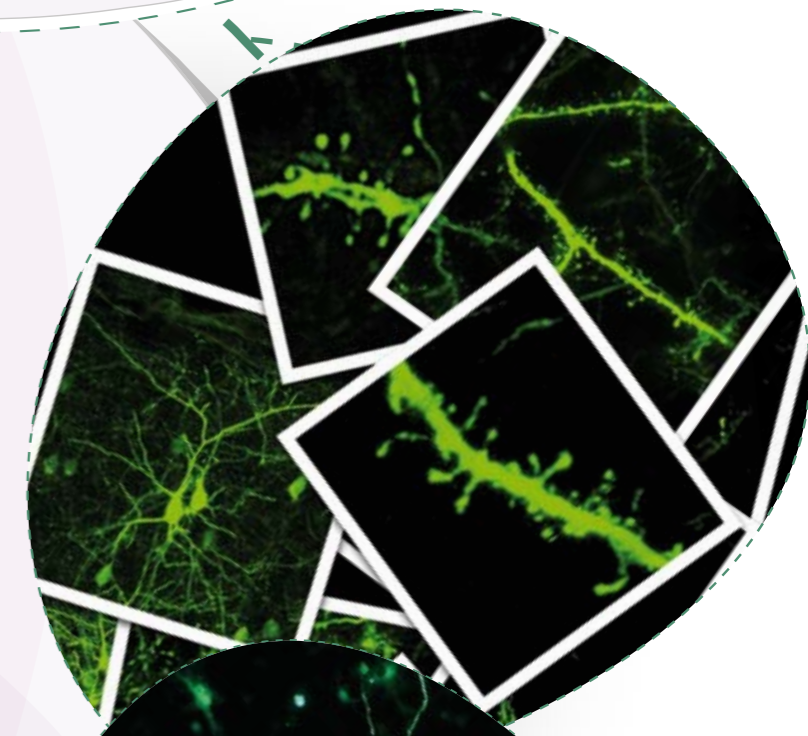
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and defects in working memory. Our work demonstrates critical roles for *Cux* in dendritogenesis and highlights novel subclass-specific mechanisms of synapse regulation that contribute to the establishment of cognitive circuits. Understanding brain wiring is an enormous task, fundamental for the dissection of normal cognitive processing and the molecular basis of disease. In particular, abnormalities in the development of layer II-III neurons are pathological, and associate to mental retardation and autism.

Dendritic structures. Green fluorescent protein (GFP)-labelled neurons and dendritic extensions generated after in utero electroporation of embryonic neural precursors.



Cellular & Molecular Biology

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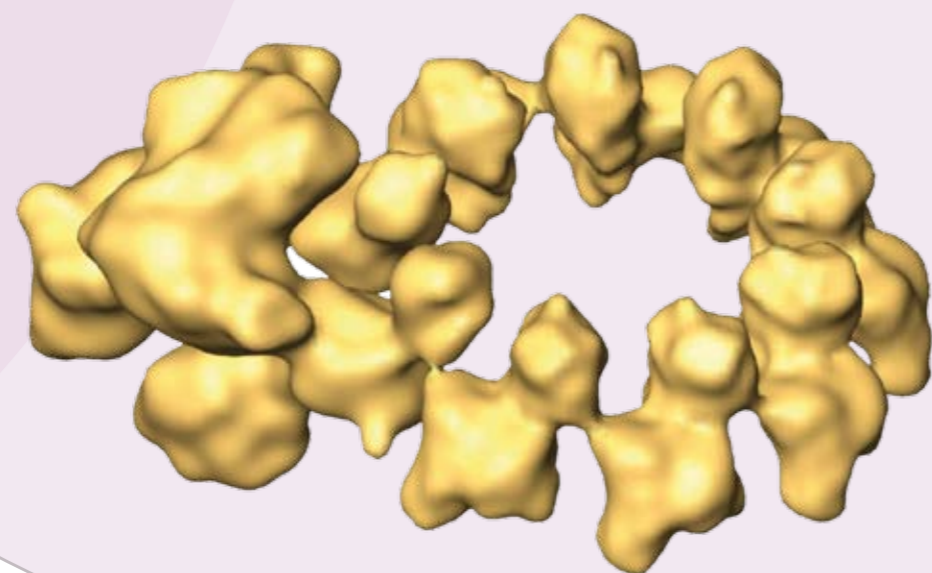
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 Marcela Benavides

Transcription and Replication of Influenza Virus RNA

In the last two years, our group has continued the study of the structure and function of the influenza virus ribonucleoproteins (RNP) and the included polymerase complex. We have purified recombinant RNP as well as viral polymerase complex expressed in human cells.

The structure of these viral complexes was studied by electron microscopy of negative-stained or frozen samples and three-dimensional reconstruction, in collaboration with Jaime Martín-Benito, José María Valpuesta (CNB) and Óscar Llorca (CIB).

On the one hand, we established the first structure of an influenza RNP by cryo-EM at 12-18 Å resolutions that describes the interactions among NP monomers as well as the interactions of NP with the polymerase complex. The structure of the polymerase contained in the RNP is the best information available so far on any negative-strand RNA virus polymerase, and has allowed the localisation of specific domains of the included subunits. In addition, we described the structure of the polymerase in association to the template RNA but devoid of NP. These complexes were generated by replication *in vivo* of a micro-RNA template and show similarities, but also differences, with the polymerase integrated in



a functional RNP. The mechanism of viral RNA replication and transcription was analysed by genetic *trans*-complementation tests. We used highly efficient procedures for the generation and purification of recombinant RNP, and also characterised polymerase mutants defective in either RNA replication or transcription, to determine whether the polymerase associated to the parental RNP is responsible for the synthesis of the progeny RNA and/or its encapsidation into progeny RNP. The results are compatible with a new model for virus replication whereby a polymerase complex distinct from that present in the parental RNP carry out RNA synthesis *in trans* and yet another polymerase complex is in charge of encapsidating the progeny RNA. In contrast, the polymerase complex associated to the parental RNP carry out RNA transcription *in cis*.

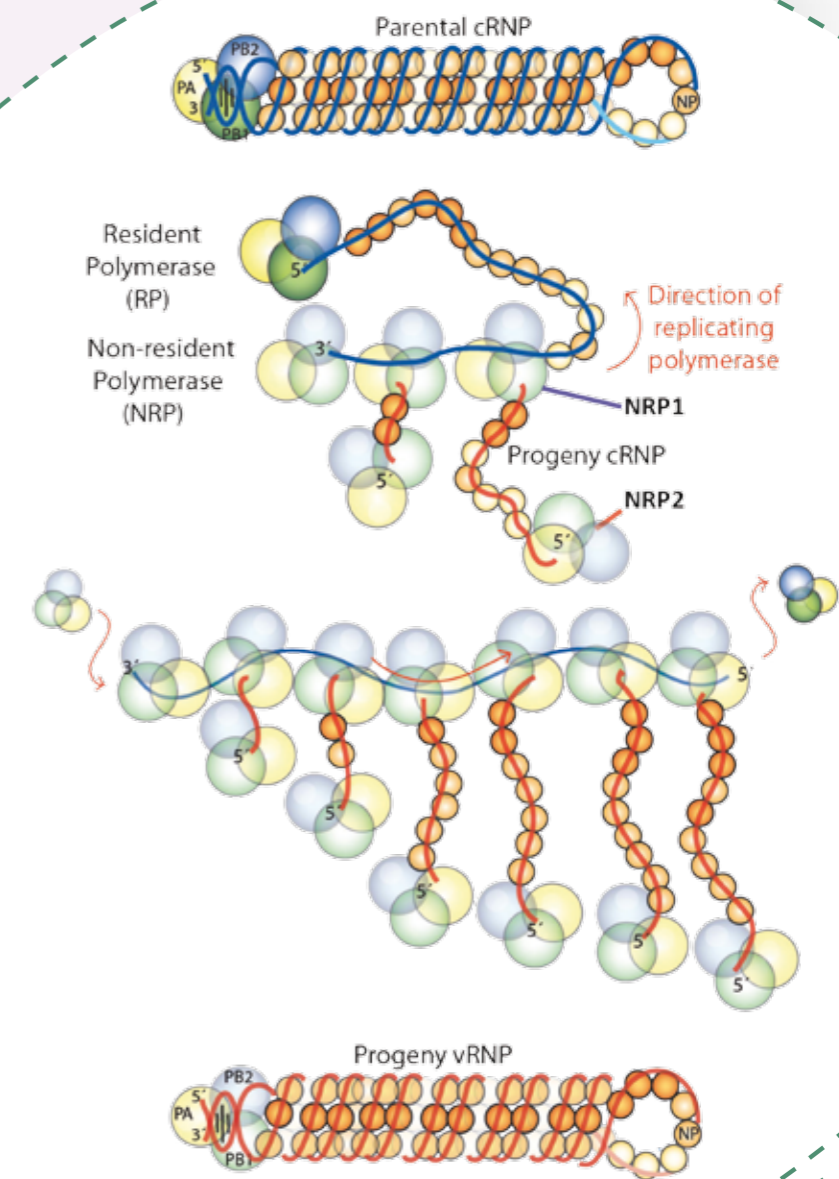
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Cellular & Molecular Biology

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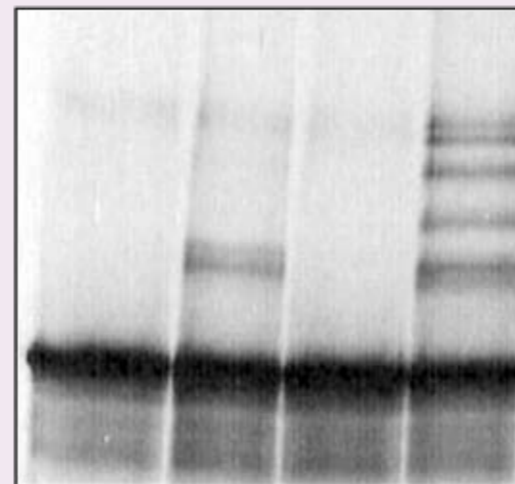
Virus and Cancer

Our group is interested in the relationship between virus and cancer.

One of our lines of investigation is “virus as the driving force of cancer”. Viral infection has long been associated with human cancer; one of the most recent links is with Kaposi’s sarcoma-associated herpesvirus (KSHV), the etiologic agent of Kaposi’s sarcoma, primary effusion lymphoma and multicentric Castlemán’s disease. The mechanisms by which KSHV infection might cause cancer are still being clarified. Most of the tumour cells in these neoplasms are latently infected with KSHV, suggesting that the latent KSHV genes are responsible for the viral pathogenesis. LANA2 is one of the latent KSHV proteins, and was shown to be absolutely necessary for survival of KSHV-infected primary effusion lymphoma cells. In recent years, our group has identified some of the functions of this viral protein, which suggest that LANA2 has an important role in the transforming activity of the virus. Continuing with these studies, in the last two years we have:

- Identified a new function that might contribute to the pathogenesis of the virus, the disruption of cell PML-NB by LANA2.

SAE1/2	+	+	+	+
Ubc ^o	+	+	+	+
SUMO-1	-	+	-	-
SUMO-2	-	-	-	+

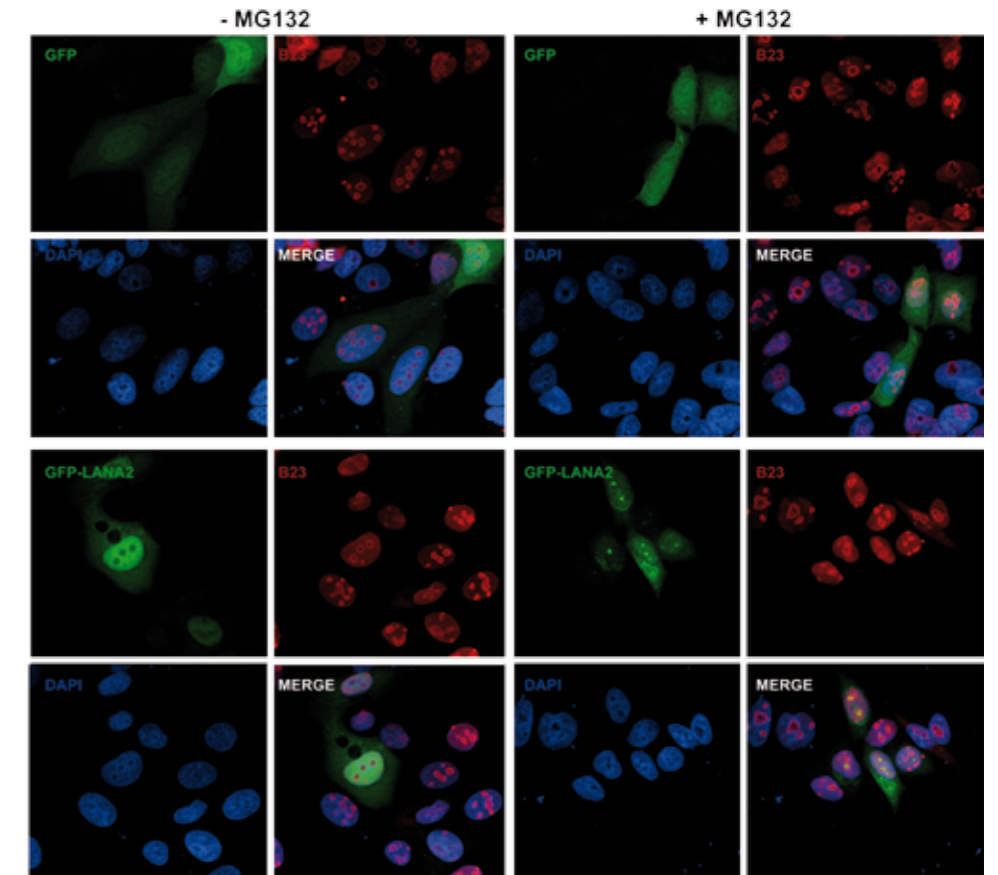


Sumoylation of LANA2 in vitro.

- Demonstrated that LANA2 activity is regulated by SUMO modification.
- Demonstrated the induction of chromosome instability by LANA2.

In addition, we are interested in evaluating the importance of different tumour suppressors in the complex innate antiviral host defence. DNA tumour viruses have developed mechanisms to inhibit tumour suppressors, and activation of some tumour suppressors after interferon treatment has been described. Together, these results suggest that tumour suppressors could be important for the antiviral response of the cell, providing new links between tumour suppression and the antiviral host defence. As a result of our studies on the regulation of virus infection by major components of the cell tumour suppression mechanisms carried out in the last two years, our group has:

- Identified the tumour suppressor pRb as necessary for correct activation of the NFκB pathway in response to virus infection.
- Described a new function of the deacetylase SIRT1: regulation of correct PML-NB formation.



Nucleolar translocation of LANA2 upon inhibition of proteasome-dependent protein degradation.

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Cellular & Molecular Biology

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Molecular Characterization and Epidemiology of Torovirus

Our general objective is to study different biological aspects of the toroviruses, emergent viruses that are practically ignored although they have the potential to infect and cause enteric diseases in various animal species and in man.



Double-membrane vesicles (DMV) in cryosections of BEV-infected cells.

Toroviruses are enveloped viruses with a single-stranded RNA genome, which belong to the *Coronaviridae* family within the order *Nidovirales*. Four genotypes or species have been identified so far: equine torovirus (EqToV or BEV), bovine torovirus (BToV), porcine torovirus (PToV) and human torovirus (HToV). The impossibility of growing these viruses in cultured cells, with the single exception of the equine torovirus BEV, has probably contributed to their insufficient study.

Our group has developed two specific assays for diagnosis of porcine torovirus (PToV): an ELISA to determine antibodies to torovirus in serum samples, and a real time RT-PCR for detection and quantitation of PToV in clinical samples.

Results using these assays show that porcine torovirus is highly prevalent in Spanish porcine livestock; this was the first study to show the presence of torovirus in Spain. We later performed a longitudinal study analysing samples taken from animals at different times during the piglet's life, which showed that they are infected shortly after weaning, when maternal protection transferred through colostrum declines. We are currently performing an extensive epidemiological study and genetic characterization of the viral strains identified.

Another research line focuses on the study of virus-host interactions. We observed that BEV causes apoptosis at late times post-infection, once the new viral progeny have been produced. In addition, we showed that the structural proteins S and M are able to trigger this process when expressed individually. Thus, the induction of apoptosis in the infected cells, specifically in the enterocytes and the crypts of the enteric tract, could be the cause of the diarrhoea exhibited by infected pigs.

A third area of interest in our group is the characterisation of the torovirus replication/transcription complexes. Positive-strand RNA viruses use virus-modified cell membranes to build their replication machineries. By electron microscopy, we observed clusters of double membrane vesicles (DMV) in the cytoplasm of cells infected with BEV, the prototype torovirus. They were observed both in thin sections of infected cells embedded in conventional epoxy resin and in cryosections that were immunogold-labelled with antibodies to double-stranded RNA. We are now determining the composition of these structures and attempting to identify the origin of the associated membranes.

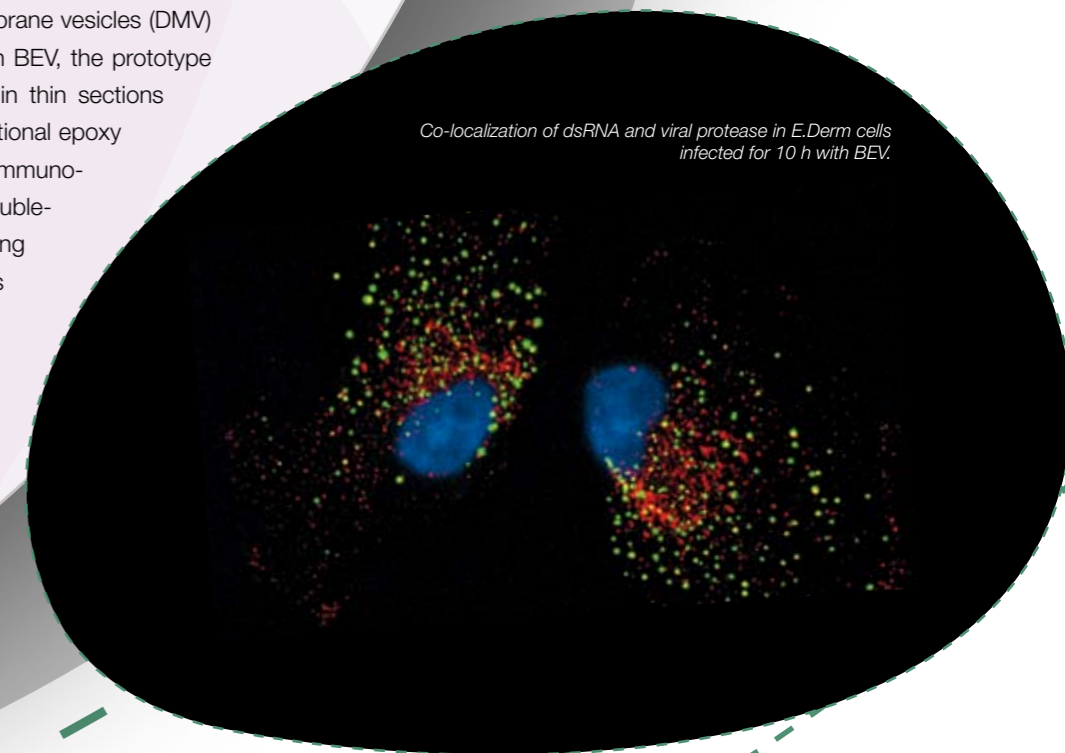
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Co-localization of dsRNA and viral protease in *E.Derm* cells infected for 10 h with BEV.



Cellular & Molecular Biology

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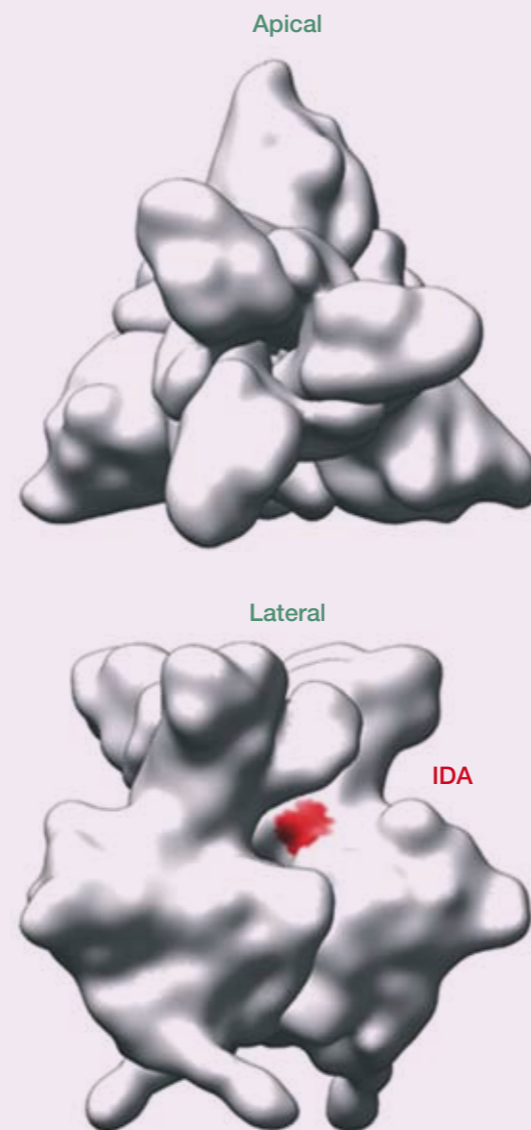
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Confocal laser scanning micrograph of QM7 cells infected with a recombinant vaccinia virus expressing a mutant version of the IBDV polyprotein encoding the capsid precursor (pVP2), the virus protease (VP4) and the scaffolding (VP3) polypeptides. Green corresponds to pVP2, Red to VP3, and Blue to nuclear staining.

Biología Molecular de Birnavirus

The Birnaviridae family groups icosahedral naked viruses with bipartite dsRNA genomes.

Members of this family infect a wide variety of animal species including insects, aquatic fauna and birds. Some of these viruses cause diseases of great socio-economic significance. Our main virus model is the infectious bursal disease virus (IBDV), the etiological agent of an acute immunosuppressive disease affecting domestic chickens. Our group is mainly interested in two broad research areas: (i) Birnavirus structure and morphogenesis; and (ii) the molecular basis for IBDV virulence.



We have continued our molecular analysis of the IBDV assembly centered on the role of the multi-functional VP3 polypeptide and the precursor of the capsid polypeptide (VP2). The characterization of the particle structure led us to demonstrate that the largest fraction of infectious IBDV particles contain four genome segments, thus providing the first evidence about the existence of icosahedral viruses with polyploid genomes. These studies led to the observation that the inner capsid space of the IBDV particles is occupied by a ribonucleoprotein (RNP) complex formed by the dsRNA genome associated to the VP3 polypeptide and the RNA dependent RNA polymerase (RdRp). The presence of birnavirus RNPs represents a sharp divergence with respect to prototypical icosahedral dsRNA viruses whose genomes remain enclosed within an inner capsid (T=2) known as transcriptional core. The unique birnavirus structural and functional configuration pose major questions about their replicative strategy, i.e. the mechanisms for RNA transcription and replication mechanisms, and those used to counteract cellular dsRNA sensors and control host-cell innate immune responses. We are currently focusing a great deal of effort to functional characterization of the VP3 polypeptide. This multifunctional and highly conserved protein that plays different roles during virus replication and morphogenesis is a key element responsible for the control of the host's innate antiviral responses.

As part of our work on virus-host interactions, we have identified the $\alpha 4\beta 1$ integrin as the major IBDV binding receptor. IBDV cell binding entails the recognition of a strictly conserved sequence motif located in the projection domain of the capsid polypeptide by the $\alpha 4\beta 1$ heterodimer. Additionally, we have shown that under certain conditions IBDV is capable of establishing persistent infections in chicken lymphocytes. These studies have opened new venues to characterize the mechanism of IBDV entry, and the molecular determinants for virus tropism, and for the development of new strategies to control IBDV infection.

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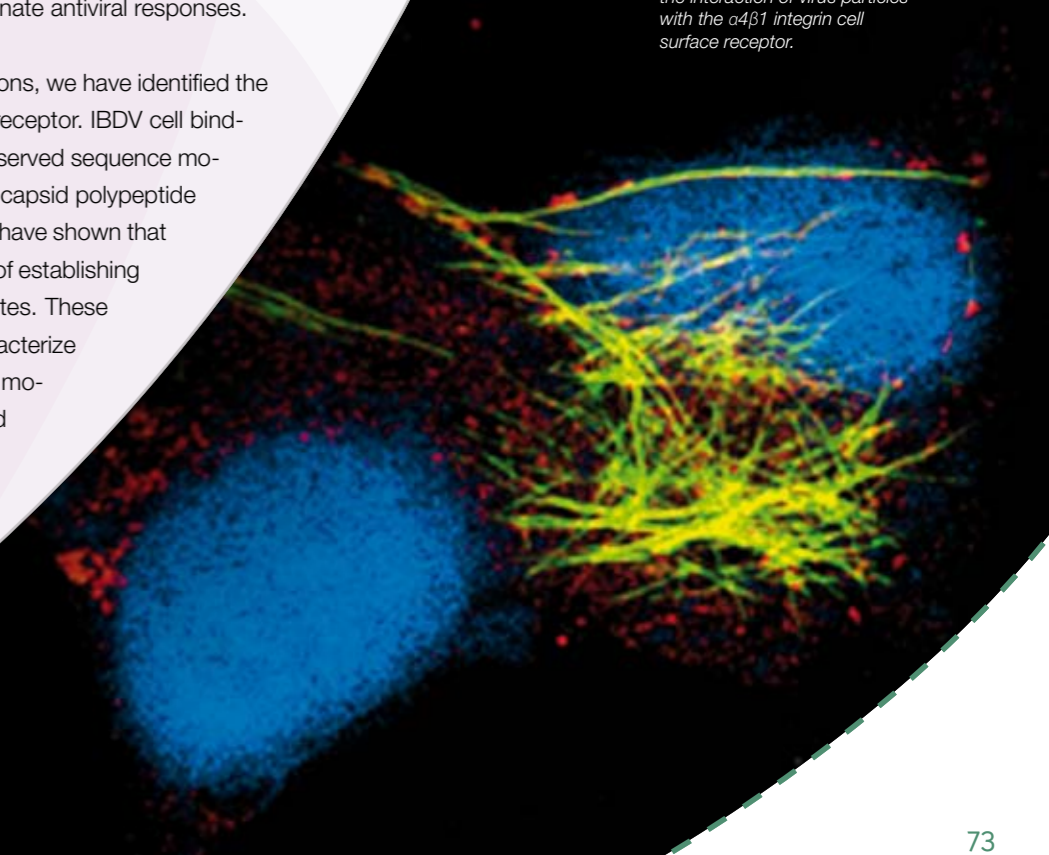
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Apical and lateral views of a three-dimensional model of the VP2 trimer, the building block of the IBDV capsid. The red path indicates the position of the "IDA" tripeptide responsible for the interaction of virus particles with the $\alpha 4\beta 1$ integrin cell surface receptor.



Cellular & Molecular Biology

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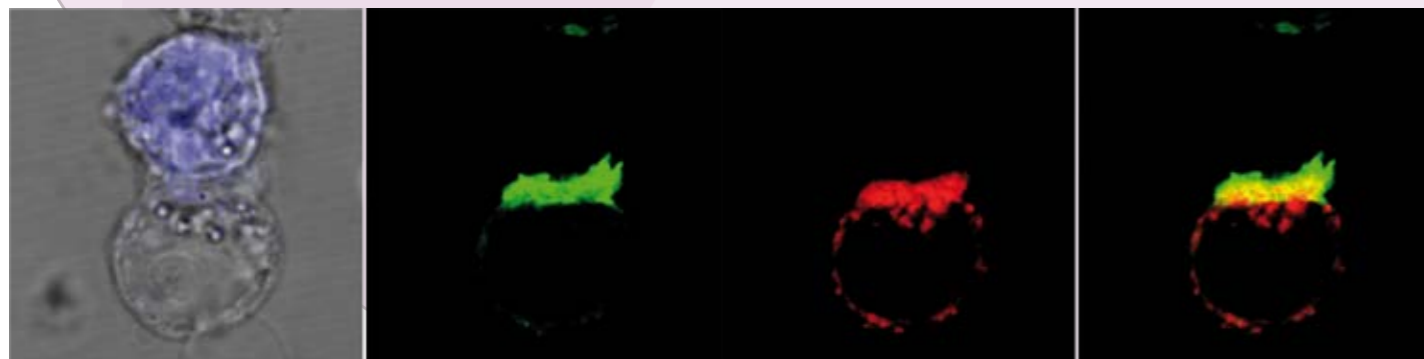
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Clathrin accumulates at the IS. Combination fluorescence and phase contrast image showing an antigen (SEE)-loaded APC (blue) conjugated with a T cell expressing td-Tomato-LCa (clathrin; red), actin-GFP (green).



Cellular Immunobiology and Microbiology

In the last two years (2009-2010), our group, “Cellular Immunobiology and Microbiology (ICM)” has been studying the molecular and cellular mechanisms that allow the formation of immunological synapses.

To become activated, T cells must establish cell-cell contact with antigen-presenting cells (APC). This contact, known as the immune synapse (IS), drives major morphological and functional changes in T cells, including massive actin rearrangements necessary for productive IS formation. The IS serves as a platform for large-scale molecular exchange between the IS-forming cells. Multiple cytokines and vesicles, which drive intercellular communication, are released to the synaptic cleft. We were the first to show that clathrin is essential for the massive actin polymerization observed at the IS. This finding, observed in cell lines and primary cells, is of outstanding relevance not only to immunologists, but also to cell biologists and scientists in the field of cellular microbiology. We are currently carrying out studies on the molecular mechanisms that drive pathogen infections, and extending these studies to the intimate relationship between bacterial and fungal pathogens with the cells of the immune system. We are also exploring the possible therapeutic use of bacterial products able to modify the immunological system.

We are currently financed by a project from the Spanish Ministry of Science and Innovation, BFU2008-04342/BMC.

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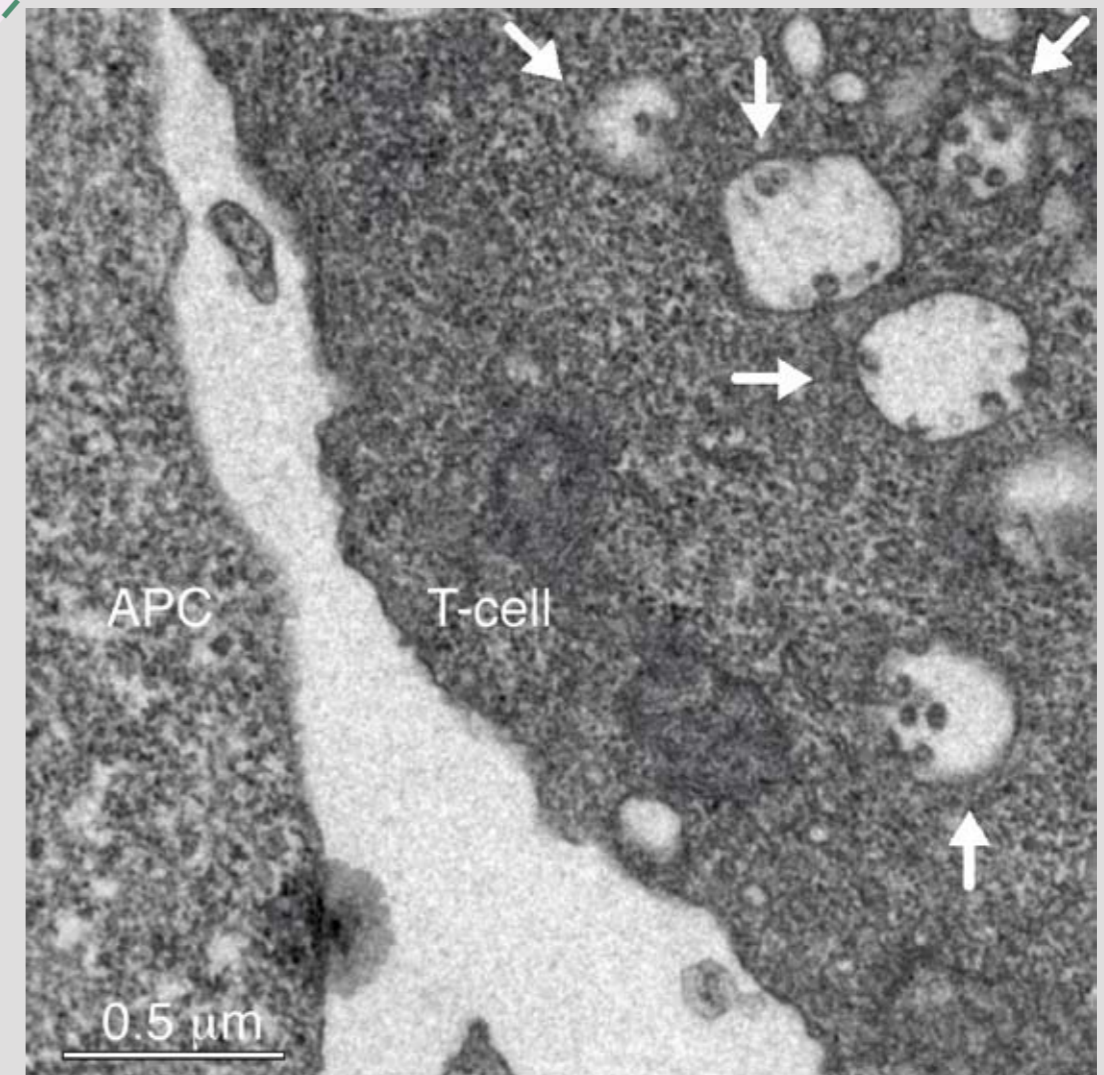
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Electron micrograph of the IS. The MVB are clearly visible near the cell-cell contact area.





Microbial Biotechnology

The Department of Molecular Microbiology integrates research aimed at gaining knowledge on key aspects of microbial biology with environmental, clinical or biotechnological relevance. Work, organised in twelve distinct lines, exploits several complementary aspects of microbial biology, with approaches that include molecular genetics, genomics, proteomics and metagenomics. The subjects studied include:

- Environmental microbiology. We aim to understand how microorganisms degrade organic compounds that generate environmental problems, as well as why a microorganism that can efficiently degrade a compound of interest under laboratory conditions does usually not perform so well under natural conditions.
- Microbial responses to hostile environments. The focus is to understand bacterial responses to stressful environments, including the reaction to host defence responses and to agents causing DNA damage. We study how opportunistic pathogens adapt to survive within a host, eliciting defence responses against the immune system, and how bacteria and fungi replicate DNA and repair DNA damages to improve genome stability.
- Microbial pathogens. Efforts are directed to understand how microbial pathogens infect or invade a eukaryotic host, with particular emphasis on the host-pathogen interactions occurring in infections caused by intracellular bacterial pathogens and phytopathogenic fungi. This will facilitate finding new targets to combat microbial diseases relevant in human health and agriculture.
- Microbial resistance to antibiotics and search for new antimicrobials. We aim to understand mechanisms of bacterial resistance to antibiotics and to analyse the complex responses elicited upon exposure of microbes to sublethal concentrations of antibiotics. In addition, we search for new antimicrobials and new potential targets in essential functions to fight against pathogens.
- Microbial engineering. The purpose is to generate bacterial strains optimised to obtain products of interest (recombinant antibodies, hydrolytic enzymes or antimicrobial compounds), or to detect and degrade pollutants. Efforts are also made towards understanding the mechanisms used by bacteria to export and secrete proteins.

These topics are highly interconnected. While most established pathogens normally cope with stressful conditions by developing efficient adaptive responses, the opportunistic pathogens are metabolically very versatile, what makes them efficient biodegraders of organic chemicals. Fighting against pathogenic microorganisms requires a deep understanding of their behaviour during infection and of how resistance develops as pathogens are challenged by antibiotics.



JUAN C. ALONSO
Genetic Stability

78

SILVIA AYORA HIRSCH
Recombination-Dependent DNA Replication

80

JESÚS BLÁZQUEZ
Stress and Bacterial Evolution

82

ARTURO CALZADA
Cell Cycle, DNA Replication and Genome Stability in Eukaryotes

84

LUIS ÁNGEL FERNÁNDEZ HERRERO
Protein Secretion and Antibody Expression

86

FRANCISCO GARCÍA-DEL PORTILLO
Intracellular Bacterial Pathogens

88

FRANCISCO MALPARTIDA ROMERO
Molecular Genetics of *Streptomyces*

90

JOSÉ PÉREZ MARTÍN
Molecular Mechanisms of Fungal Virulence

92

JOSÉ LUIS MARTÍNEZ
Opportunistic Pathogens

94

RAFAEL P. MELLADO
Heterologous Gene Expression and Secretion in Gram-positive Bacteria of Industrial Application

96

FERNANDO ROJO
Regulation of the Metabolism of Hydrocarbons in Bacteria

98

MIGUEL VICENTE
Genetic Control of the Cell Cycle

100

Microbial Biotechnology

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Genetic Stability

Our aim is to characterise the involvement of DNA repair and segregation in the stability of the genetic material in Firmicutes using two model systems.

Bacillus subtilis is used to study repair-by-recombination, and plasmid pSM19035 to study segregational stability. In the first model, we showed that i) during double-strand break repair, RecN in concert with polynucleotide phosphorylase (PNPase) promotes the dynamic recruitment of DNA ends. PNPase distributively removes few nucleotides from the 3'-end, ii) AddAB or RecJ, in concert with a RecQ-like helicase (RecQ or RecS), creates 3'-ssDNA tails at the break, and RecN recruits recombination proteins to form a repair centre, iii) various mediators (e.g., RecO, RecU) modulate the activity of the recombinase RecA, iv) the RecO mediator mediates second end capture (Fig. 1), and v) the branch migration helicases (RecG and RuvAB), and the resolvase (RecU) process recombination intermediates.

In the second model system, we showed that i) the role of pSM19035 global regulator ω protein (a dimer in solution, ω_2) is to control the fine-tuning of plasmid copy-number, plasmid segregation, and ϵ and ζ expression. In addition, ii) the partitioning protein ω_2 binds to a centromeric-like region, forming a left-handed protein matrix surrounding the straight parS DNA (partition complex, PC), and protein δ (a dimer in solution, δ_2) hydrolyses ATP preferentially in the presence of ω_2 bound to parS (segrosome complex, SC) or non-parS DNA (dynamic complex, DC) and binds DNA after interacting with ω_2 , promoting plasmid pairing (bridging complex, BC) at parS DNA or pseudo bridging complex ("BC") at non-parS DNA. Finally, iii) the δ_2 disassembly from DNA is dependent on the stoichiometry of the ω_2 : δ_2 complex.

The different stages are summarized in Fig. 2.

Scientific Report 09-10
Centro Nacional de Biotecnología CNB

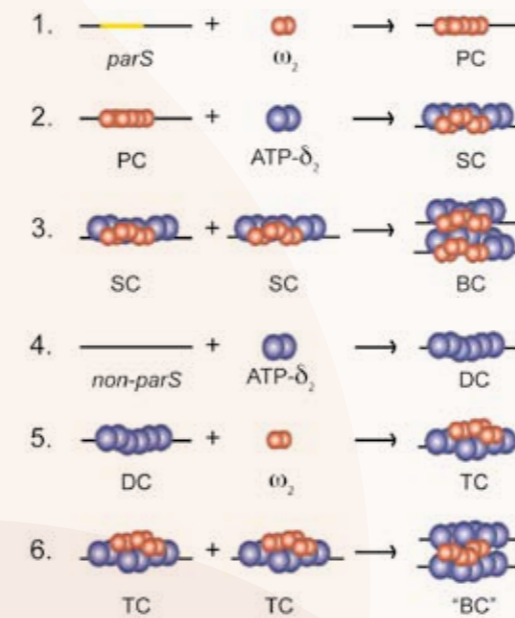


Fig. 2. Dynamic assembly of different types of protein-DNA complexes. Protein ω_2 bound to parS DNA led to the formation of a partition complex (PC, 1); δ_2 bound to PC led to segrosome complex (SC, 2) formation, and the interaction of two SC led to bridging complex (BC, 3) formation. Protein δ_2 bound to any DNA (non-parS DNA) led to dynamic complex (DC, 4) formation; ω_2 binding to DC led to a transient complex (TC, 5), and the interaction of two TCs led to pseudo-bridging complex ("BC", 6) formation.

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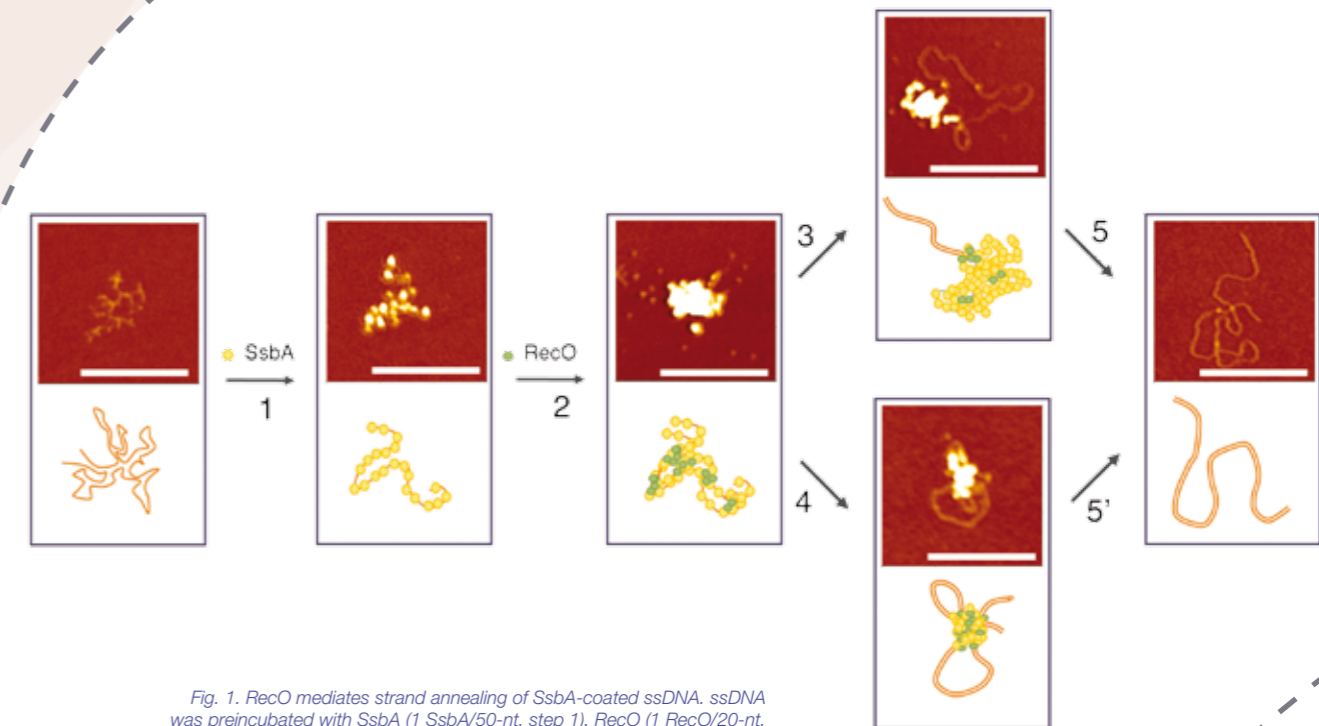


Fig. 1. RecO mediates strand annealing of SsbA-coated ssDNA. ssDNA was preincubated with SsbA (1 SsbA/50-nt, step 1). RecO (1 RecO/20-nt, step 2) was added and the reaction incubated for a variable time (steps 3 to 5'). In steps 3 and 4, a partial RecO-mediated dislodging of SsbA leads to RecO-promoted homology search and strand annealing. In steps 5 and 5', RecO-mediated strand annealing leads to the naked dsDNA product. Samples were taken at different times. Schematic representation of the images is shown below. Scale bar = 200 nm.

Microbial Biotechnology

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Recombination-Dependent DNA Replication

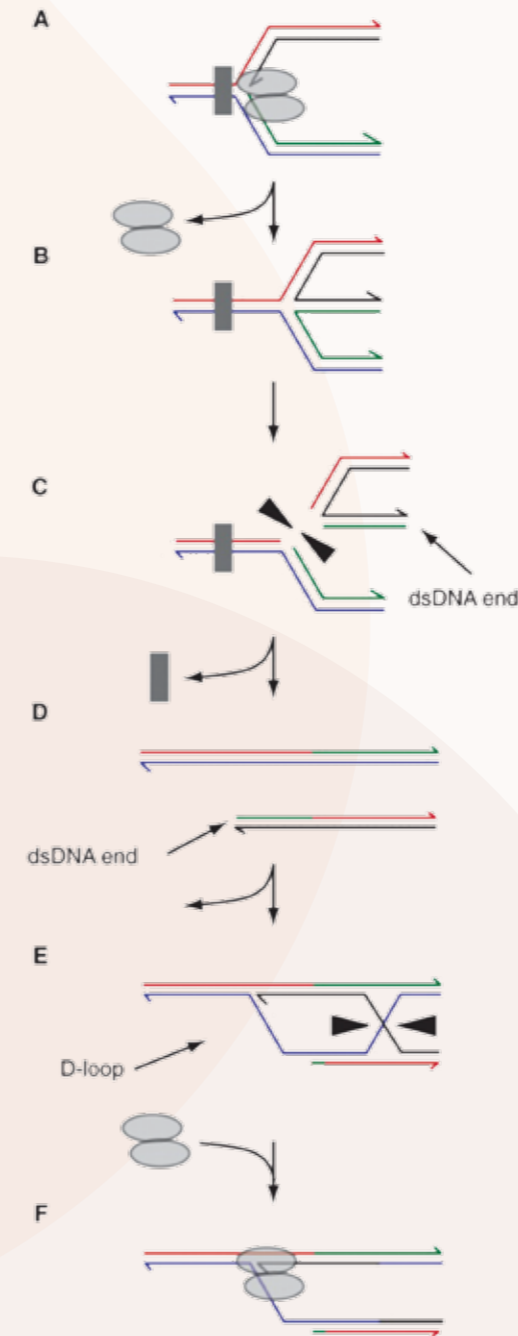
A central problem in biology is the faithful transmission of hereditary information from mother to daughter cells.

This process not only involves precise replication of chromosomal DNA, but also correct partitioning of the newly synthesized sister chromosomes. It has become clear over the last decade that the progression of replication forks in living cells and their viruses is disrupted with high frequency, by encountering various obstacles either on or in the DNA template. Survival of the organism then becomes dependent both on removal of the obstacle and on restart of DNA replication. Homologous recombination is a process that takes place in all living cells to generate diversity, DNA repair, and for correct segregation of the chromosomes. It is also necessary to properly reassemble the arrested replication fork (Fig. 1). The study of homologous recombination mechanisms has revealed the complexity of the recombination process, due to the large number of proteins involved. Simple model systems such as bacteria and their viruses (bacteriophages) are therefore good candidates for deciphering these complex mechanisms.

We are analyzing how a replication fork is reassembled, and how replication can restart by a recombination-dependent mechanism, using *Bacillus subtilis* and its bacteriophage SPP1. In these two models, the outcome of the recombination-dependent replication is different, as in the bacteria the product is replication restart by a theta mechanism, whereas in SPP1, replication restart leads to concatemeric DNA synthesis which is the substrate for viral DNA packaging. The latter type of replication is also found in herpes simplex virus, baculovirus, mitochondrial DNA, chloroplast DNA, telomeric circles, and certain pathogenicity islands, and takes place by a poorly characterized mechanism.

In the *B. subtilis* work, we focus mainly on the RecU Holliday junction (HJ) resolvase and the RecU modulators RuvA and RuvB (Fig. 2). This enzyme has three activities: (i) it cleaves HJ, (ii) anneals complementary strands and (iii) modulates RecA

Scientific Report 09-10
Centro Nacional de Biotecnología CNB



SELECTED PUBLICATIONS

Carrasco B, Cañas C, Sharples GJ, Alonso JC, Ayora S (2009) The N-terminal region of the RecU Holliday-junction resolvase is essential for homologous recombination. *J Mol Biol* 390:1-9.

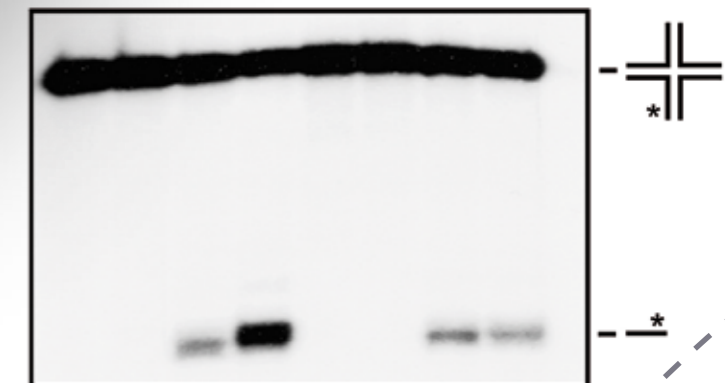
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Restart of DNA replication by fork regression and Holliday Junction (HJ) formation. A) The replisome is stalled. B) The replisome disassembles and the fork regresses. C) HJ resolvases recognise the four-strand intermediate and cleave it. D) The lesion/block is removed, and the dsDNA end processed. E) The resulting end invades a homologous duplex (by the action of a recombinase). The four-strand recombination intermediate is resolved by HJ resolvases. F) The replisome assembles on the new fork.

activities. It interacts with the RecA recombinase and with the RuvB branch migration helicase. We have mapped the region essential for this interaction (1), and observed that RecU is recruited to the competence machinery, probably for modulating RecA activities at the competence pole (2).

	RecU	-	-	100	100	100	100	100	100	nM
RecU	-	-	100	100	100	100	100	100	100	nM
RuvB	-	40	-	40	-	40	-	40	-	nM



RuvB-ATPS-Mg²⁺ fails to stimulate RecUR31A-HJ cleavage, and RecUΔ1-32 is incapable of HJ cleavage. HJ were assembled from four oligonucleotides (80 nt in length), one of which was radiolabelled; the cleavage activity of RecU variants was assayed in the presence of RuvB on a denaturing gel.

Microbial Biotechnology

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Stress and Bacterial Evolution

The major interest of the group is to understand the bacterial responses to stress.

We specifically study hypermutation and hyperrecombination as bacterial "strategies" to speed adaptation to environmental stresses. One of the models used here is antibiotic stress and the development of antibiotic resistance. Our work focuses on both stable and inducible hypermutation/hyperrecombination in *E. coli*, *P. aeruginosa* and *M. smegmatis/tuberculosis*.

We are currently studying:

1. Compensation of stable hypermutation. Once adapted, hypermutable bacteria must decrease (compensate) the high mutation rate to avoid accumulation of deleterious mutations. Our study aims to unveil the molecular mechanisms involved in such compensation.
2. Regulation of stress responses and inducible hypermutation
 - Environmental regulation of mutagenesis
 - Transcriptional regulation of specialized DNA polymerases (belonging to the SOS regulon)
 - Effect of antibiotics on mutation and recombination. Antibiotics as promoters of antibiotic resistance.
3. Hypermutation in bacteria lacking mismatch repair system (MMR) such as *Mycobacterium* and *Streptomyces*. This will allow the use of hypermutant/hyperrecombinant bacteria of industrial interest as biotechnological tools to produce modified biosynthetic pathways.
4. Evolution of resistance to β -lactam antibiotics and the development of new inhibitors of β -lactamases.
5. Molecular bases of bacterial evolution. Combatting antibiotic resistance by preventing evolution (mutation, recombination and horizontal transfer).



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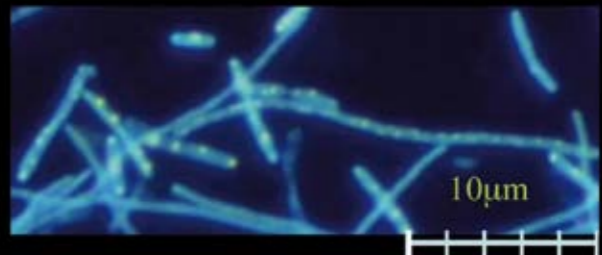
No CAZ (3-5h)



CAZx2 (3h)



CAZx2 (5h)



Filamentation of *P. aeruginosa* cells produced by antibiotic treatment. Antibiotic stress elicits morphological, transcriptional and adaptive changes.

Microbial Biotechnology

LEAD INVESTIGATOR
Arturo Calzada

Cell Cycle, DNA Replication and Genome Stability in Eukaryotes

We study the regulation of eukaryotic DNA replication.

Defects in the replicative machinery often result in DNA damage and/or genome instability. Our approach is to characterize the molecular abnormalities arising in DNA replication caused by mutations in replication regulators naturally associated to tumorigenesis, to unveil new regulatory pathways and to understand how disease is produced.

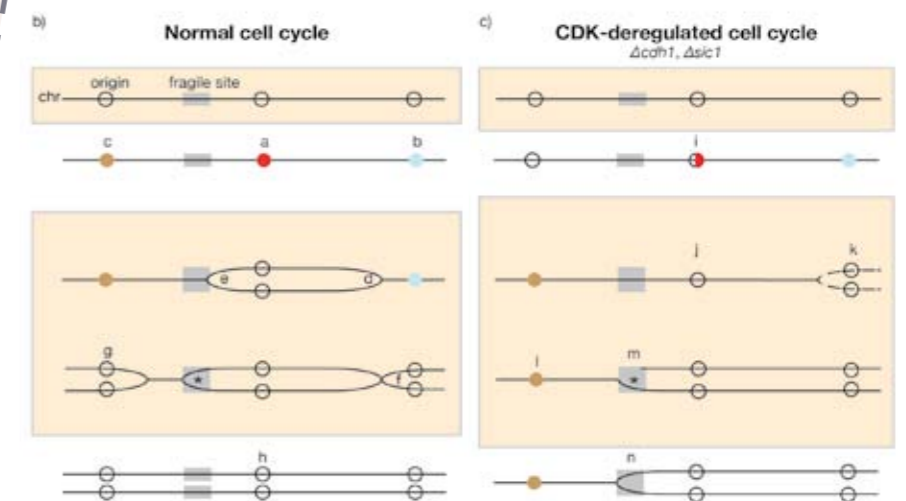
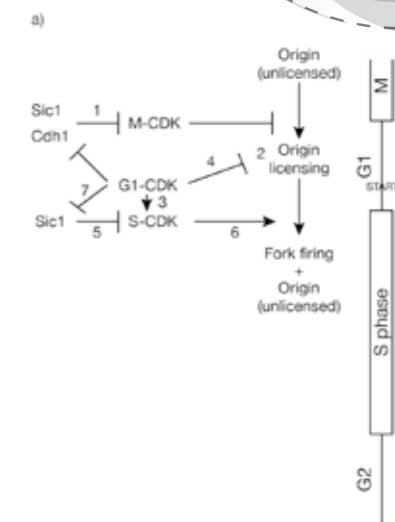
Cyclin-dependent kinase (CDK) complexes regulate the initiation of DNA replication, activating fork firing at origins of DNA replication at the G1/S transition, and inhibiting origin licensing to ensure a complete, unique replication per cell cycle. Absence of CDK in G1 is essential for optimal origin licensing. CDK upregulation in G1 causes genome instability and is oncogenic. Recent work by others in yeast and human cells show that CDK upregulation in G1 induces an abnormal, lengthened S phase, with increased DNA damage including double strand breaks. One obvious consequence of increased CDK activity in G1 is reduced origin licensing and firing, as described for a few origins in yeast. What is molecularly different from normal in S phase,

how and where damage is produced, and how it induces genome instability, is poorly understood. We approach these questions in *Saccharomyces cerevisiae* yeast cells, which have proved useful in cell cycle and DNA replication studies.

CDK/cyclin B complexes are maintained inactive in G1, mainly by cyclin degradation of mitotic cyclins by ubiquitination dependent on Cdh1-APC/C (anaphase-promoting complex/cyclosome) and CDK/cyclin inhibition by Sic1 (orthologue to p27 in animal cells) (1, numbers indicate the detail in Fig. 1a), so origin licensing is allowed (2). At start, G1 cyclins are synthesised to activate G1-CDK (3), which in turn activates S-CDK (4), and inhibits licensing (5). Sic1 inhibits S-CDK (6) activity, necessary for fork firing at G1/S (6), until Sic1 degradation is promoted by G1-CDK (7). Our results with cells lacking Sic1 and/or Cdh1 show, first, that most replication origins studied maintain normal efficiency (resistant origins), and only some (30%) lose efficiency (sensitive origins) to a different extent (Fig. 2, ARS507 and ARS508). Second, origin sensitivity is independent of normal origin firing timing during S phase, origin activity, or origin location on chromosomes, as observed in contiguous origins. Third, cells do not compensate the reduction in forks by firing of silent origins (Fig. 2). Fourth, the rate of gross chromosomal rearrangement (GCR) increases close to the sensitive origin ARS507 (Fig. 2). All these results suggest that when CDK is deregulated, chromosome regions proximal to sensitive origins are more prone to genome instability. Whether GCR occur by attempts to segregate partially unreplicated chromosomes or by fork progression impediments is unknown. Results are summarized in Fig. 1c, in comparison with a normal cell (Fig. 1b).

SELECTED PUBLICATION

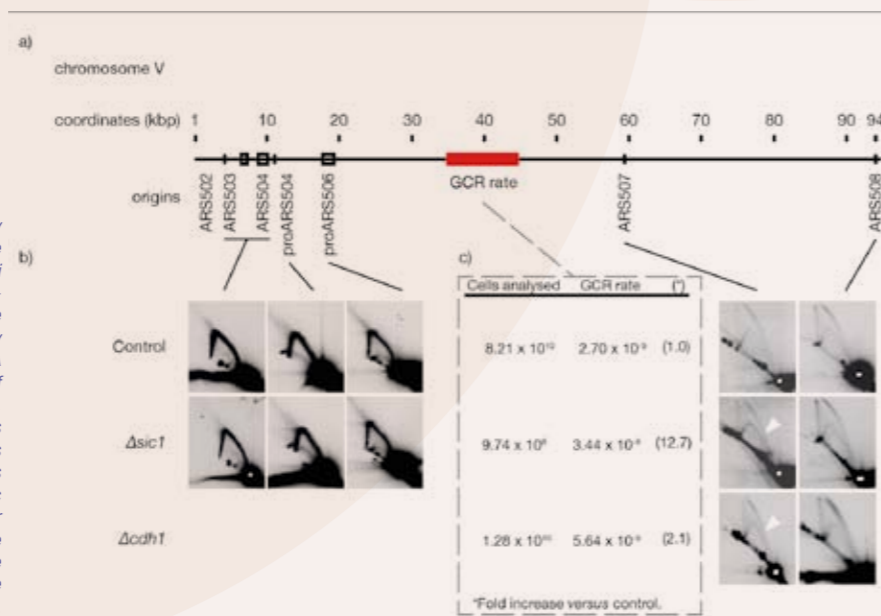
Gambus A, van Deursen F, Polychronopoulos D, Foltman M, Jones R, Edmondson RD, Calzada A, Labib K (2009) A key role for Ctf4 in coupling the MCM2-7 helicase to DNA polymerase alpha within the eukaryotic replisome. *EMBO J* 28:2992-3005.



CDK regulation in G1 is important for efficient fork firing at a subset of sensitive origins to prevent chromosome rearrangements in its proximity. a) CDK regulation from M exit to S phase in *S. cerevisiae* cells, in origin activation. Numbers are explained in the text. b) and c), models resuming data from figure 2. b) In normal cells, early (a) and late (b) firing origins, and silent origins (c) are licensed in G1. In S phase, early origins initiate two divergent forks migrating (d) or pausing (e) depending on chromatin features. Late origins fire later in S phase (f). If fork collapse occurs (g), silent origins fire (g) to avoid unreplicated sequences after S phase ends. Replication render origins unlicensed (h), cl In CDK deregulated cells, licensing in G1 is incomplete at sensitive origins (i), probably due to reduced G1 length either by inefficient M-CDK inhibition or premature S-CDK activation. Unfired origins (j) change the replication dynamics at that particular region that is now replicated by forks fired at resistant proximal origins (k). Silent origins do not fire to compensate unfired origins (l). Chromosomal rearrangements increase in the area normally replicated by a CDK-deregulated sensitive-origin, possibly as a consequence of DNA breaks (m) or regions left unreplicated in S phase (n). Open circles, unlicensed origins. Closed circles, licensed origins.



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Replication dynamics and chromosomal stability at the left arm of chromosome V in *S. cerevisiae* cells. a) left arm of chromosome V including coordinates from the telomere, and location of replication of real (ARS503, 504, 507, 508) and putative (proARS504, 506) origins. b) Origin activity by two dimensional agarose electrophoresis of DNA to measure origin efficiency, where the intensity of the external arc indicates when the origin is active, and the internal arc indicates when the origin is inactive; comparison of both arcs determines origin efficiency. A white arrowhead indicates when the origin loses efficiency (the internal arc gains signal, and the external arc loses signal) for cells of the strains on the left. c) Estimation of the Gross Chromosomal Rearrangements rate for the indicated strains (Chen and Kolodner, 99, *Nature Genet.* 23,81-85).

Microbial Biotechnology

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Protein Secretion and Antibody Expression

Our group conducts both **basic and biotech-oriented research** aimed to understand and exploit protein secretion in *E. coli* and other **Gram-negative bacteria** (proteobacteria).

Our basic research focuses on the molecular mechanisms that bacterial pathogens employ for the secretion of proteins (e.g. cytotoxins, proteases, adhesins) and for the assembly of cell surface organelles (e.g. fimbriae) that participate in bacterial virulence. We focus especially on those proteins and surface organelles secreted and assembled by **pathogenic *E. coli* strains** like enteropathogenic (EPEC), enterohemorrhagic (EHEC) and uropathogenic (UPEC) *E. coli* strains. The biotechnological projects exploit these protein secretion systems for the development of novel **expression and selection technologies for recombinant antibodies** in non-pathogenic commensal and laboratory *E. coli* strains. Among the recombinant antibody formats available (e.g. single-chain Fv, Fab, Fc-fusions, etc.), we focus on single-domain antibodies (**sdAbs**) or **nanobodies**, the smallest antibody fragment known-to-date (~15 kDa) with full antigen-binding capacity. Nanobodies are based on a single V_H domain obtained by recombinant DNA technology from heavy-chain-only antibodies from camelids (e.g. dromedaries, llamas). Despite the lack of a paired V_L domain, nanobodies show high-affinity and specificity for their cognate antigens. In addition they are highly similar to human V_H3 sequences, making them excellent candidates for multiple applications, including human therapy.

Some of our current projects are:

The secretion mechanism of the bacterial type V secretion system (T5SS) and its application for the bacterial display of single-domain antibodies. The T5SS include proteins with "self-translocation" capacity across the outer membrane like the Intimin-Invasin family and the so-called autotransporters (ATs), which are the major family of proteins secreted by Gram-negative bacteria. ATs have distinct biological functions important during the pathogenesis of the producer

microorganism (e.g. proteolysis of host proteins and antibodies, cytotoxicity of host cells, adhesion to host tissue, etc). In addition to investigate the secretion mechanism of ATs, we are exploiting the translocator domains of T5SSs to display nanobodies on the surface of *E. coli* for selection of binders against specific antigens (bacterial display).

Assembly of type 1 fimbriae. Type 1 fimbriae are thin proteinaceous filaments assembled on the surface of *E. coli* cells by the ordered polymerization of a major protein subunit (FimA) and several minor protein subunits (FimF, FimG, FimH) assembled by the chaperone-usher pathway. Type 1 fimbriae and FimH adhesin are essential for effective colonization and invasion of the epithelial cells of the urinary bladder by UPEC strains. We found that the N-terminal lectin domain of FimH is recognized by the fimbrial usher FimD in order to initiate the assembly the adhesin and the polymerization of the fimbrial filament. The mechanism of activation of FimD by the N-lectin domain of FimH is currently under investigation.

Injection of nanobodies from *E. coli* into human cells. We are employing the type III protein secretion system (T3SS) from EPEC and EHEC strains, to directly deliver single-domain recombinant antibodies from *E. coli* cells into the cytosol of human cells. During infection T3SSs act as molecular syringes for the translocation of proteins from bacteria into eukaryotic cells. *E. coli* injection of nanobodies does not require bacterial invasion of the eukaryotic cell or the transfer of any genetic material.

Structure of conventional and heavy-chain only antibodies, comparing the structure of different recombinant antibody fragments, including single chain Fv (scFv) and single-domain antibodies (sdAbs).

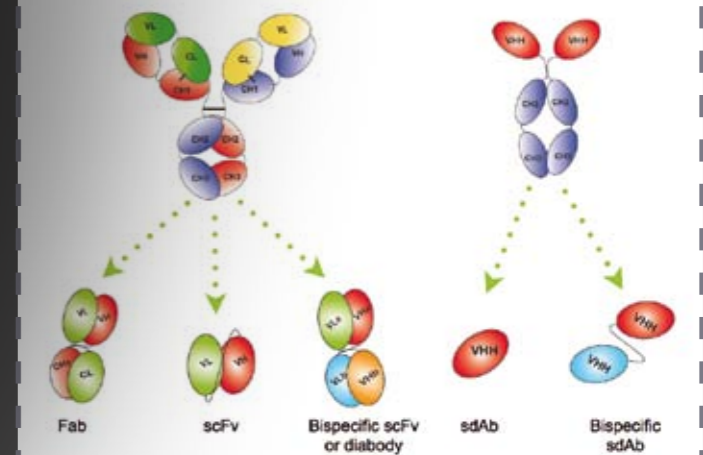
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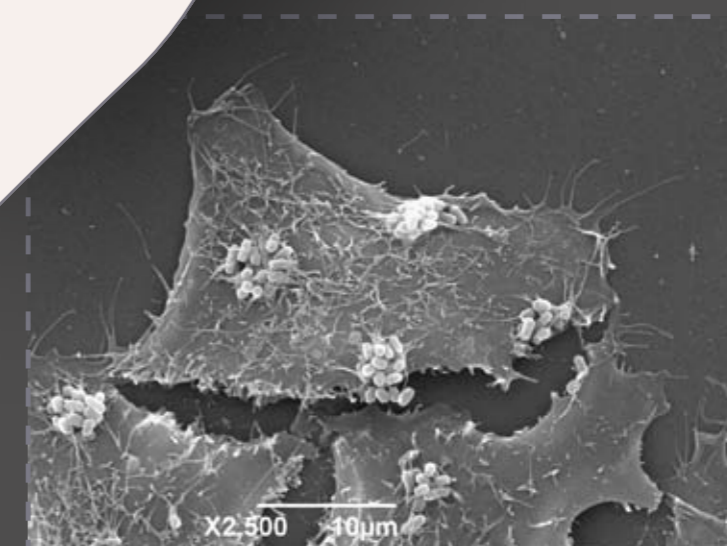
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Antibody fragments



Scanning electron micrograph of human HeLa cells infected in vitro with attenuated EPEC bacteria carrying a functional T3SS injecting sdAbs into the cytoplasm of the human cell.



Microbial Biotechnology

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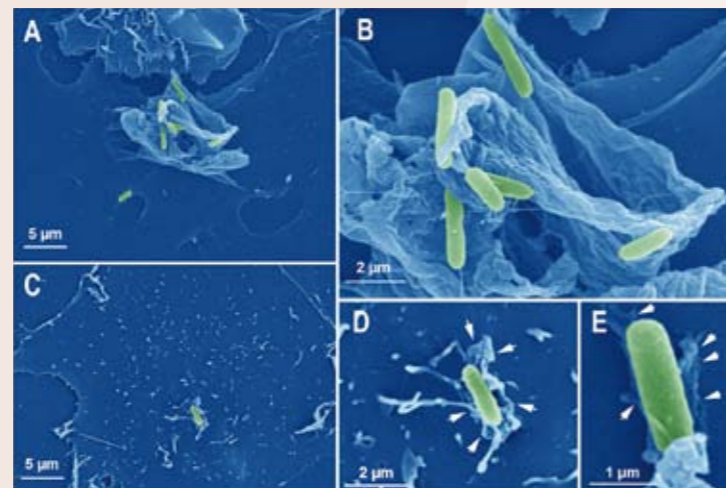
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Scanning electron microscope images denoting the massive membrane remodelling concomitant to the entry of wild-type *Salmonella* into NRK49F rat fibroblasts (A-B). Entry of the Δ SPI-1 mutant takes place by a zipper-like mechanism involving discrete filopodia and lamellipodia (C-E) (adapted from Alastui et al. *Infect Immun* (2010) 78:2700).



Intracellular Bacterial Pathogens

Using *Salmonella* and *Listeria* as models, our group attempts to decipher the lifestyle of bacterial pathogens inside eukaryotic cells and the host responses to these intracellular infections.

We have progressed in the characterization of small regulatory RNA expressed by non-growing intracellular *Salmonella* inside cultured fibroblasts.

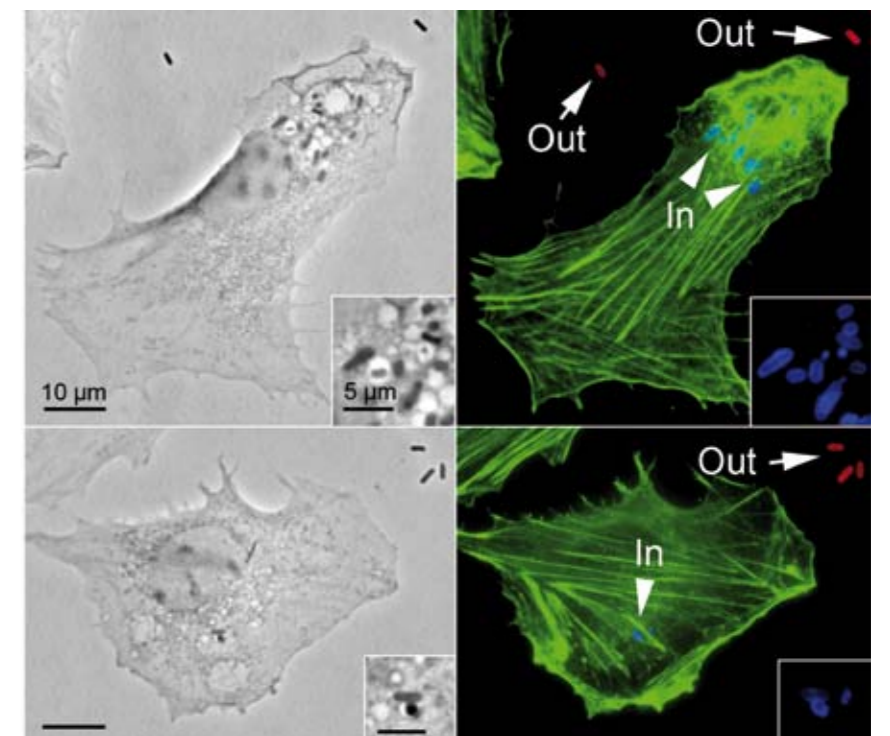
Some of these sRNA were found to be upregulated in intracellular bacteria only at late post-infection times. This expression pattern suggests that a subset of sRNA might play a role in long-adaptation of intracellular bacteria to limited nutrient availability and stress. A novel sRNA was also identified in the virulence plasmid pSLT. This sRNA, named 0995, is expressed at high levels by non-growing intracellular bacteria. Current effort is focused in defining the targets of these sRNA and the underlying regulatory mechanisms. We also characterized, at the molecular level, the entry process of *Salmonella* into fibroblasts. This process differs from that described for epithelial cells. A representative feature was the dispensability of the GTPase RhoG for the actin cytoskeleton remodelling that promotes bacterial entry into fibroblasts. We also found differences in the extent to which membrane ruffling is elicited and the

strict requirement for the type III secretion system encoded in the *Salmonella*-pathogenicity island 1 (SPI-1).

Listeria monocytogenes surface proteins associated to the cell wall. In our effort to characterize the biological role of the *L. monocytogenes* surface protein family containing a C-terminal LPXTG motif, we continued the proteomic analysis of cell wall material isolated from bacteria growing in different conditions. We purified enough material from bacteria growing inside eukaryotic cells and identified the members of this large protein family that are synthesized by intracellular bacteria. In collaboration with other European laboratories, we also completed the series of isogenic mutants deficient in each of the 41 LPXTG proteins encoded in the genome of the *L. monocytogenes* strain EGD-e. Cell wall proteome studies in each of these mutants revealed that lack of certain LPXTG proteins impairs the activity of the sigma-B (SigB)-dependent regulon. These data connect cell wall integrity with the phosphorylation cascade that dictates the physiological state of the SigB protein. Current efforts are directed to dissecting the exact mechanisms connecting the function of this group of LPXTG proteins to the activity of the SigB regulon. In addition, we defined the features of the sorting motif recognized in the two proteins substrates of the alternative sortase of *L. monocytogenes*, SrtB.

wild-type

Δ SPI-1



Actin rearrangements triggered by *Salmonella enterica* serovar Typhimurium in NRK49F rat fibroblasts. Entry into fibroblasts of bacteria lacking the *Salmonella*-pathogenicity island 1 (Δ SPI-1) takes places without major actin remodelling.

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Microbial Biotechnology

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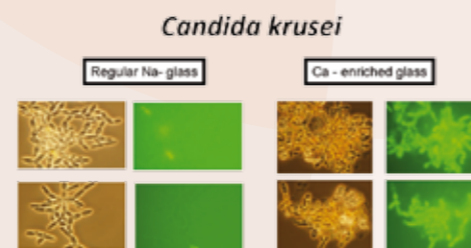
Leticia Esteban Tejeda
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Molecular Genetics of *Streptomyces*

Our group is currently focused on characterization of bioactive compounds with antimicrobial activities.

Materials from two different sources were analyzed: antifungal compounds of biosynthetic origin (polyene macrolides) and inorganic material with a broad antimicrobial spectrum (nano-structured mineral composites).

In recent last decades, systemic fungal infections are becoming a serious and increasing threat to human health. Unlike the antibacterials, the available arsenal of antifungal drugs is very limited and thus, new chemical structures with antimicrobial activities are urgently needed. The isolation of biosynthetic genes leading to bioactive metabolite production has proved to be a valuable tool for designing new chemical structures by genetic/metabolic engineering of microbial strains producers of complex bioactive metabolites. The polyene macrolides have been consid-



Bioactive polyene structures isolated from recombinant *S. diastaticus* var. 108 strains. Some polyene structures were deduced to be present in the fermentation broths (?).

ered in recent decades as secondary metabolites with excellent antifungal activity; amphotericin B is undoubtedly the "golden" drug of this family. Nevertheless, the use of this drug in clinical therapy, as well as of other polyenes, is restricted due to their associated cytotoxicity. Aimed at reducing such severe side effects, several approaches were taken for generating new and safer derivative drugs

from these old bioactive compounds: chemical synthesis to produce semisynthetic derivatives, new formulations for safely delivering drugs during systemic infection, and genetic manipulation of biosynthetic genes to generate new derivatives from previous bioactive drugs. By targeting genes of the rimocidin biosynthetic pathway and the tailoring activity of a carboxamide synthase from *S. diastaticus* var. 108, ten new polyene derivatives were isolated from these recombinant strains.

In addition to organic compounds of biosynthetic origin, several inorganic materials were analyzed as antimicrobial agents. Microbial contamination is not only a serious threat that compromises human and animal health; several industrial processes are also affected by uncontrolled growth of microorganisms. In some environmental conditions, the use of biocides of biosynthetic origin can be unreliable for controlling undesirable microbial populations; the inorganic antimicrobials seemed to be, at least intuitively, more active and stable in those circumstances. In the last few years, several bioactive materials have been tested against a large range of microbial cultures, including nano-structured materials of defined composition. Most are very abundant in nature and possibly suitable for use in many socio-economic areas, particularly when efficiency as a biocide, chemical stability and environment protection are needed.

Micrographs of an oxonol-guided antimicrobial test. The green fluorescence of the oxonol indicator shows the failure of the electrochemical gradient across the membrane. The yeast culture was treated with microparticles of regular windows glasses (left) or Ca⁺⁺-enriched glasses particles.

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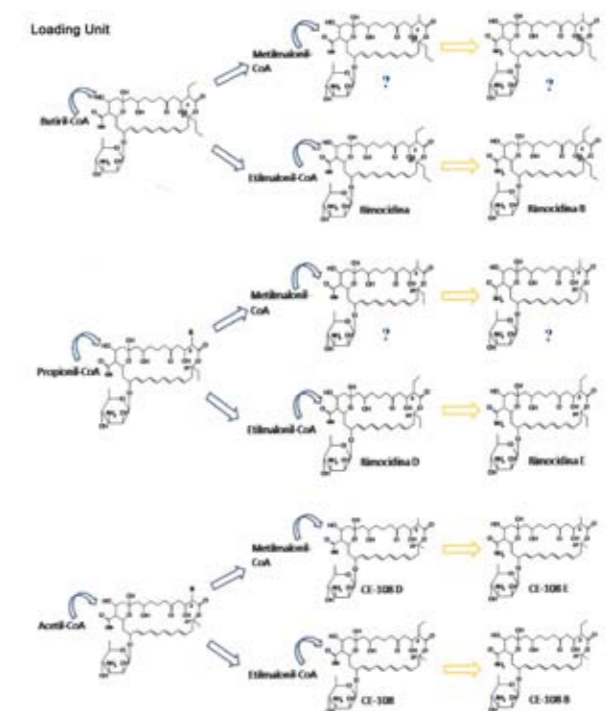
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Esteban-Tejeda L, Malpartida F, Esteban-Cubillo A, Pecharrómán C, Moya JS (2009) Antibacterial and antifungal activity of a soda-lime glass containing copper nanoparticles. **Nanotechnology** 20: 505701.

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J. Moya, L. Esteban, C. Pecharrómán, F. Malpartida. Polvo de composición vítrea con actividad biocida. **P200931137**.



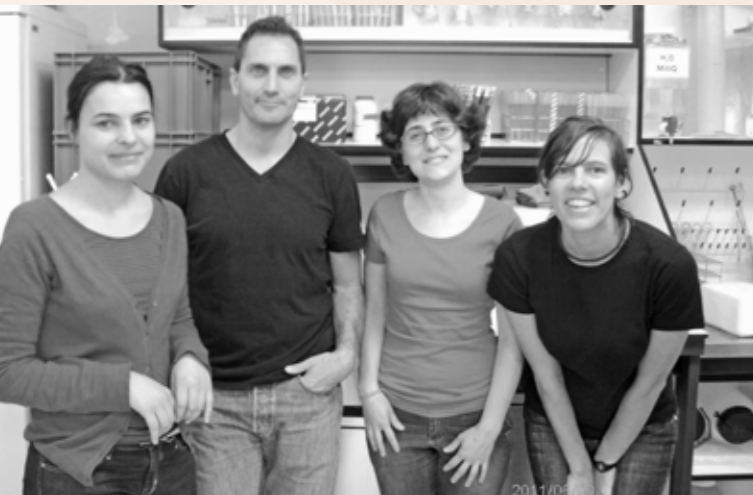
Microbial Biotechnology

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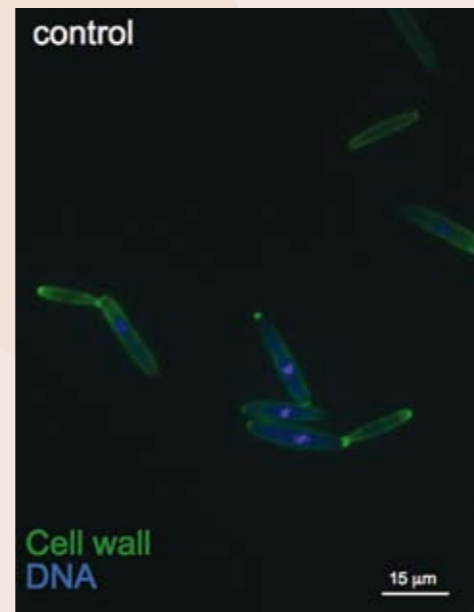
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Molecular Mechanisms of Fungal Virulence

Our group hypothesised that cell cycle regulation would be likely to provide control points for infection development by fungal pathogens.

We consider that the cell cycle has to be adjusted in response to both environmental and developmental signals, and that the integration of both classes of signals by the cell machinery will result in an outcome that define fungal fate: in the case of pathogenic fungi, whether



Activation of the cell wall damage pathway induces strong morphological changes. Cells expressing a constitutive kinase (*mkk1^{DD}*) display morphological defects (additional details in Carbo & Perez-Martin 2010).

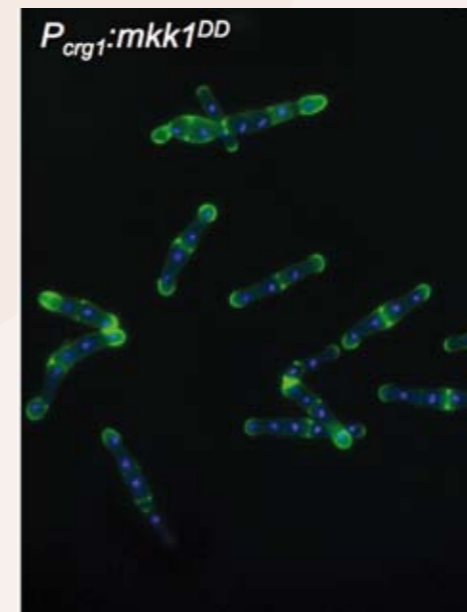
or not they enter the virulence program. The corn smut fungus *Ustilago maydis* is an excellent system in which to address these relationships between cell cycle, morphogenesis and virulence. Induction of the virulence program in this fungus implies not only strong morphological changes (bud to hypha transition) but also genetic changes (haploid

Scientific Report 09-10
Centro Nacional de Biotecnología CNB

to dikaryotic transition). Consequently, accurate control of the cell cycle and morphogenesis is predicted during these transitions.

To achieve the specific aims of this hypothesis, the scientific objectives have been framed in two major groups. The first group of objectives addresses how the cell cycle is modified during the pathogenic phase, whereas the second addresses the importance of morphogenetic pattern in the cell cycle responses to environmental signals transmitted by MAPK cascades.

Our research offers original opportunities to address the molecular basis of fungal virulence from a different point of view, which at the same time is complementary to previous approaches in the field more focussed on the study of signal transduction and transcriptional changes.



SELECTED PUBLICATIONS

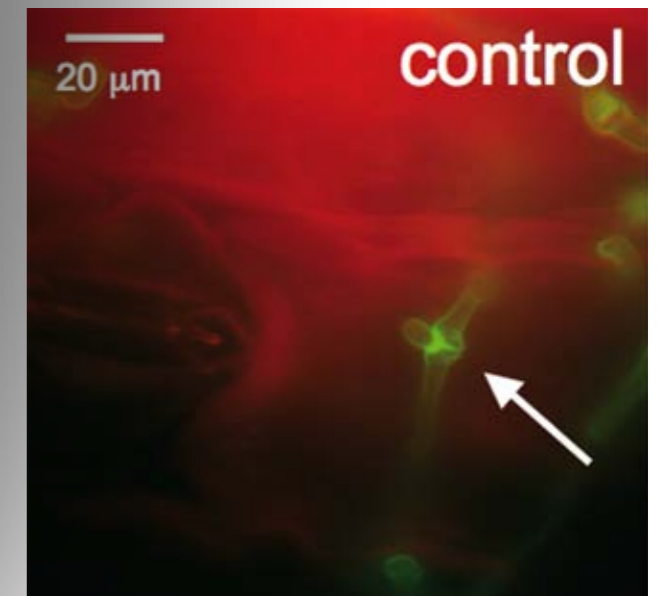
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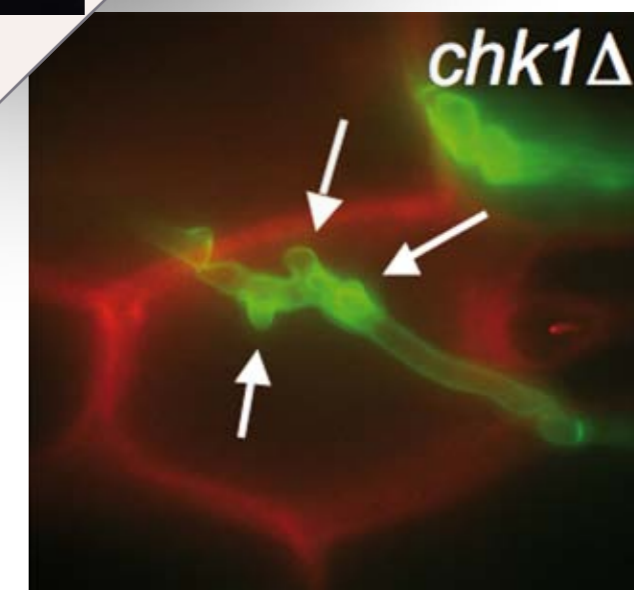
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The DNA damage pathway is required for in planta proliferation. Mutant fungi lacking the checkpoint kinase Chk1 proliferate aberrantly in corn plants (additional details in Mielnichuk et al. 2009).



Microbial Biotechnology

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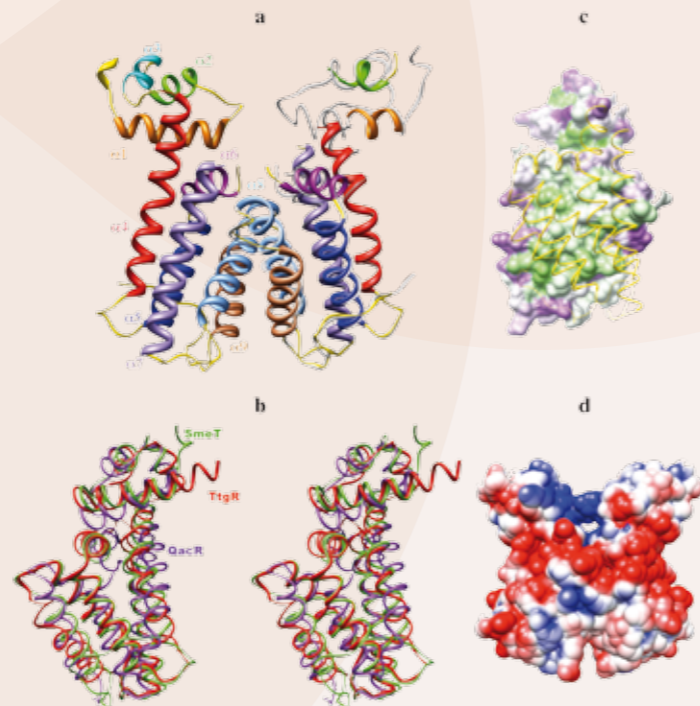
Opportunistic Pathogens

Infectious diseases remain among the major causes of human death in the world.

Several infections at hospitals are due to opportunistic pathogens, microorganisms that rarely infect healthy people, but are a frequent cause of infection in people with basal diseases, who are immunodepressed or debilitated.

Environmental bacteria, frequently antibiotic resistant, constitute a large percentage of those pathogens. Our work focuses on understanding the mechanisms of virulence and resistance, as well as their possible crosstalk, of these pathogens.

Within this scope, in the last two years, we have been defining those genes whose mutation changes the phenotype



Structure of SmeT. 1a) Ribbon representation of SmeT; helices 1-9 are shown in different colours and are labelled only in subunit A. Subunit A is superposed onto subunit B and is shown in grey. 1b) Stereo view of the structural comparison between SmeT (green), QacR (magenta, ID code 1JUP) and TtgR (red, ID code 2UXU). The extended N and C termini of SmeT are shown. 1c) Conservation of the hydrophobic character of the residues in the interface of the SmeT dimer. The figure was prepared using HOMOLMAPPER, based on alignment of 288 sequences from members of the TetR family prepared by M-Coffee. Residues in green have a conserved hydrophobic character; residues in magenta do not. Subunit B is represented as a yellow backbone tube to show the dimerisation area. 1d) Electrostatic surface potential of the SmeT dimer. Since the model is incomplete, subunit A was superposed onto subunit B and the dimer thus generated was used for calculations. Electrostatic potential was calculated by APBS and is mapped on the solvent-accessible surface by CHIMERA. Electrostatic potential values range from positive (blue: 5 kT/e) to negative (red: -5 kT/e) (from Hernandez et al. 2009. J Biol Chem 284:14428).

of antibiotic susceptibility of *Pseudomonas aeruginosa*. As a result, we have selected nearly three hundred genes for future analysis and are currently studying whether those mutations that challenge intrinsic resistance also alter the virulence of *P. aeruginosa*. We found that mutations in several genes encoding proteins from different categories that include multidrug efflux pumps, two component systems, metabolic enzymes or global regulators, simultaneously alter the antibiotic susceptibility and the virulence of *P. aeruginosa*. One of these is Crc, a global regulator involved in *Pseudomonas* carbon metabolism. We found that a *crc*-defective mutant is more susceptible to several antibiotics and expresses several virulence determinants at lower levels than the wild-type strain. These results indicate that the resistance to antibiotics and the virulence of *P. aeruginosa* are intrinsically linked to bacterial metabolism.

Another opportunistic pathogen we are working with is *Stenotrophomonas maltophilia*, which is characterized by its intrinsic low susceptibility to several antibiotics. Part of this low susceptibility relies on the expression of chromosomally-encoded multidrug efflux pumps. Expression of *smeDEF* is downregulated by the SmeT repressor, encoded upstream of *smeDEF* in its complementary DNA strand. We determined the crystal structure of SmeT and analysed its interactions with its cognate operator. SmeT behaves as a dimer and presents some common structural features with other TetR regulators. At difference from other TetR proteins for which the structure is available, SmeT turned out to have two extensions at the N and C termini that might be relevant to its function. *In vitro* studies showed that SmeT binds to a 28 bp pseudopalindromic region, forming two complexes. This operator region overlaps the promoters of *smeT* and *smeDEF*, a finding consistent with a role for SmeT in simultaneously downregulating *smeT* and *smeDEF* transcription.

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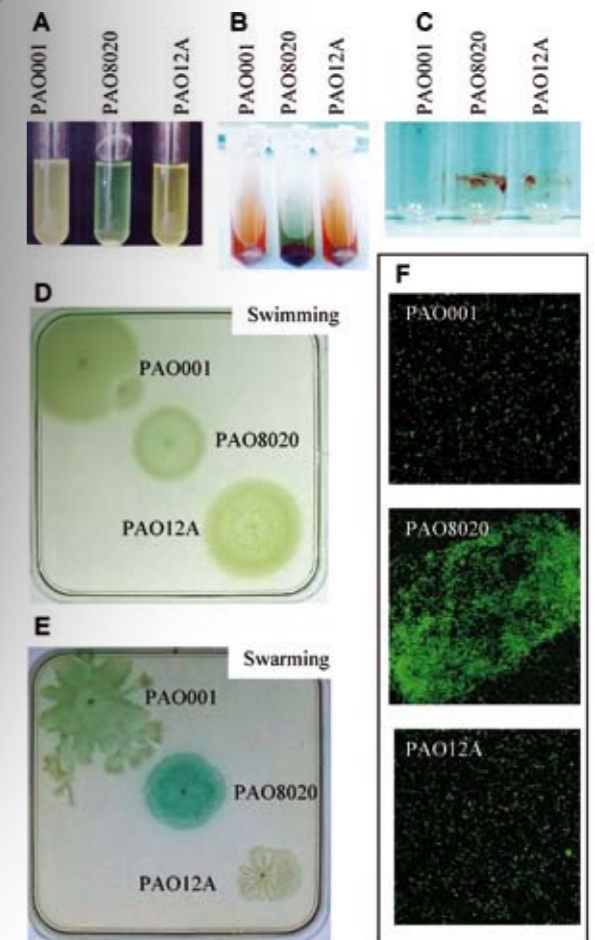
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Effect of Crc on the multicellular behaviour of *P. aeruginosa*. In all panels, PAO001 is the wild-type strain, PAO8020 is the *crc* mutant and PAO12A corresponds to PAO8020 containing a plasmid encoding Crc. A) As shown, the *crc* mutant overproduced the green phenazine pyocyanine, attaches to the air-liquid interface and forms large bacterial aggregates (see bottom of tube containing the *crc* mutant). Phenotypes are restored after complementation in PAO12A. B) Cells were grown in LB medium, stained with Congo Red and centrifuged. Congo Red is retained only by the *crc* mutant PAO8020 pellet, indicating EPS production by this strain. C) Cells attached to the walls of culture tubes were stained with Congo Red to study the presence of exopolysaccharides in the multicellular structure. D, E) Effect of Crc on *P. aeruginosa* motility. Lack of *crc* impaired both swimming (D) and swarming (E) motility of *P. aeruginosa*. Swimming motility was fully restored after expression of plasmid-encoded Crc (strain PAO12A), whereas for swarming, complementation with Crc restored formation of the protrusions typical of this type of movement (although colony size was not fully restored). F) *P. aeruginosa* aggregation was visualized by confocal microscopy. Lack of *crc* induced strong bacterial clumping. The phenotype was fully restored after expression of plasmid-encoded Crc (from Linares et al. 2010. *Environ Microbiol* 12:3196).

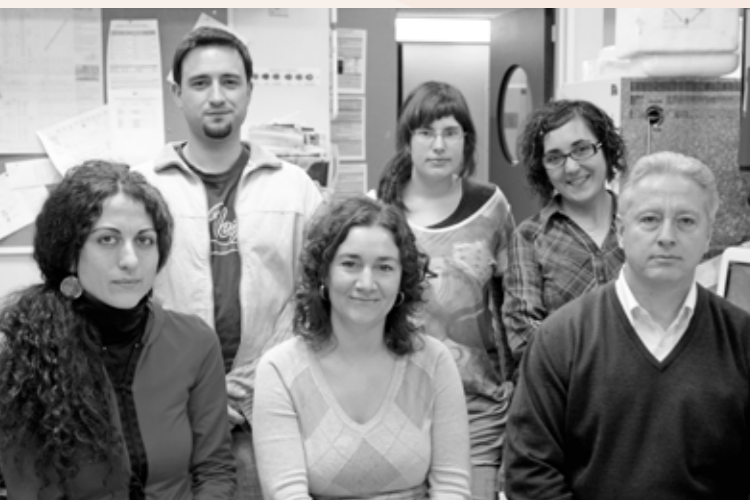
Microbial Biotechnology

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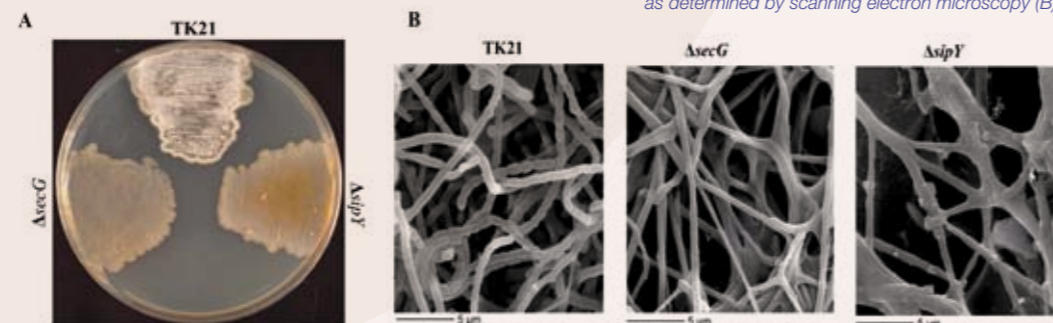
Silvia Marín

Heterologous Gene Expression and Secretion in Gram-positive Bacteria of Industrial Application

The group has traditionally focused its research on the physiological and molecular characterisation of the main protein secretion mechanism (Sec system) of the soil Gram-positive bacteria of the *Streptomyces* genus, namely *S. lividans* and *S. coelicolor*.

These are widely used in industry as efficient producers of extracellular hydrolytic enzymes and other compounds of industrial interest. Deficiency in the translocase complex (SecG mutant strain) or the major type I signal peptidase (SipY mutant strain) function in *S. lividans* results in a group of genes seemingly regulated in the same way when the translocation process of extracellular proteins is restricted, or when extracellular protein processing is compromised, including the absence of secretory protein production in both cases and a sporulation deficient bald phenotype. Transcriptional profiling and proteomic analyses of both *S. lividans* strains revealed a set of equally regulated *bld*-related genes. A bald phenotype is acquired in both mutant strains by downregulation of *bld* chain genes. Although a common regulator has not yet been identified, most of the commonly regulated genes can be linked directly or indirectly to the *bld* cascade, strongly suggesting its involvement in the cell response to the secretory defect of both mutant strains.

Sporulation deficiency. *SecG* and *SipY* deficient strains fail to sporulate in R5 medium (A), being unable to differentiate their aerial hyphae into spores, as determined by scanning electron microscopy (B).



A *S. coelicolor* two-gene operon encodes a two-component system: a histidine-kinase sensor and a response regulator protein. Propagation of the regulatory gene in high copy number results in overproduction of several extracellular proteins, as well as eliciting a partial stringent response and an altered pattern of antibiotic synthesis in the bacterial cell, as determined by proteomic and transcriptomic analyses. This two-component system is currently under study in *S. coelicolor*, as it seems to influence various processes characterised by the transition from primary to secondary metabolism.

The laboratory has started a new research line focused on molecular monitoring of the rhizobial communities of transgenic plants. Rhizobacterial communities of transgenic maize engineered to express the *Bacillus thuringiensis* Cry toxin (Bt maize) have been monitored for a number of years in three agricultural soils, using commercially available DNA microarrays containing genome-wide spotted oligonucleotides that encompass the soil bacteria *B. subtilis* and *S. coelicolor*. The results showed that genome-wide DNA arrays could be a useful tool for molecular monitoring of rhizobacterial communities to assess potential environmental risk associated with cultivation of transgenic plants.

We compared the effect of glyphosate (RoundupPlus), a post-emergency applied herbicide, and HarnessGTZ, a pre-emergency applied herbicide, on the rhizobacterial communities of genetically modified NK603 glyphosate-tolerant maize. The potential effect was monitored by direct amplification, cloning and sequencing of soil DNA encoding 16S rRNA, rhizobacterial DNA hybridisation to commercially available genome-wide microarrays from the soil bacterium *S. coelicolor*, and high throughput DNA pyrosequencing of the bacterial DNA coding for the 16S rRNA hypervariable V6 region. The results strongly suggest that both herbicides do affect the maize rhizobacterial communities, with glyphosate being, to a great extent, the environmentally less aggressive.

SELECTED PUBLICATIONS

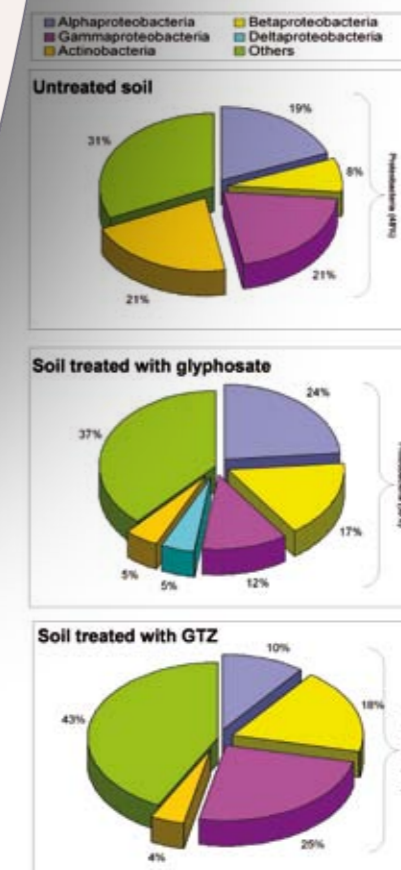
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PATENT

Rafael P. Mellado. Nueva quitinasa de origen bacteriano con amplio espectro fungicida. **PCT/ES2009/07035**. International. 2009. CSIC

Taxonomic breakdown of the more relevant phyla from the three soils seeded with maize NK603. Left: The percentages of Actinobacteria and different members of the Proteobacteria phyla are indicated. The total percentage of Proteobacteria from each soil is indicated in the right margin. Right: The herbicide treatment (lower panel) does not allow weed growth (upper panel).



Microbial Biotechnology

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Genetic Control of the Cell Cycle

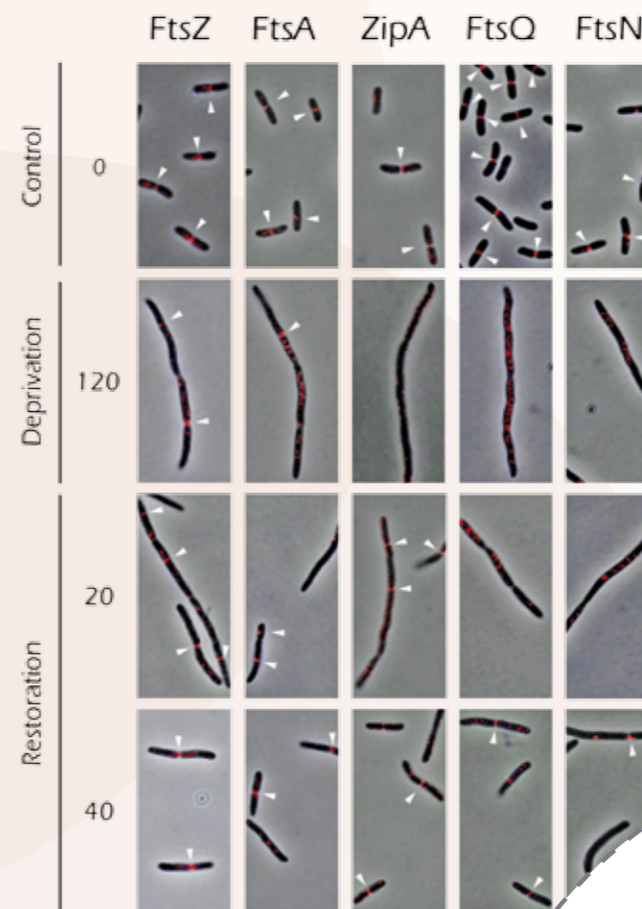
This group works to find inhibitable targets in essential bacterial functions, namely cell growth and division, with the purpose of designing assays to identify new antimicrobials.

We first study the proliferation of a Gram-negative bacteria, *Escherichia coli*, of which there are both commensal and pathogenic strains. We extend our research to study specific topics related to the proliferation of two pathogens, *Streptococcus pneumoniae*, the causative agent of serious infections, and *Mycobacterium tuberculosis*, a widespread pathogen that affects one third of the world population. We summarise two studies published in the last two years.

In the assembly of the *E. coli* divisome, we found that FtsN is required for the correct assembly and stability of the *Escherichia coli* proto-ring. The proto-ring is formed by three essential proteins, FtsZ, FtsA and ZipA. This structure is the first that assembles at midcell to initiate the division ring that, once completed, integrates into the divisome and effects cell division. FtsN, considered the last division protein to be recruited into the divisome, is required for proto-ring stability in the living cell. In its absence, the already-formed rings of FtsQ, FtsA, ZipA and even FtsZ disassemble. The ZipA in the proto-ring is the most sensitive to the decrease in FtsN levels (Rico et al., 2010. *Mol Microbiol* 76:760-771).

In collaboration with P. Gómez-Puertas (CBMSO), we studied the need for potassium and neutral pH to attain the optimal GTPase activity and polymerisation of FtsZ, the prokaryotic orthologue of tubulin. We used molecular dynamics simulations of the *Methanococcus janaschii* FtsZ dimer in the presence of GTP-magnesium and monovalent cations. The presence of potassium at the GTP binding site allows the positioning of one water molecule, promoting dimer stability and GTP hydrolysis. Sodium destabilizes the dimer and does not allow positioning of the water. Simulation of low pH by protonation of the gamma phosphate of GTP indicated that both the monovalent cation and the water molecules are excluded from the binding site. The simulation therefore

predicted that at low pH, the FtsZ GTP-ase activity is lower and the dimer more stable. These predictions were tested experimentally and shown to be correct by analyzing the GTPase and polymerization activities of *M. janaschii* and *E. coli* FtsZ proteins in the test tube (Mendieta et al., 2009. *J Mol Biol* 390:17-25).



Kinetics of ring disappearance when bacteria are deprived of the FtsN protein. Each column shows the image for each kind of ring as indicated. The images in the two bottom rows show the restoration of FtsN in the cells. Proteins were developed by immunostaining with specific fluorescently-labelled antibodies (from Rico et al., 2010. *Mol Microbiol* 76:760-771).

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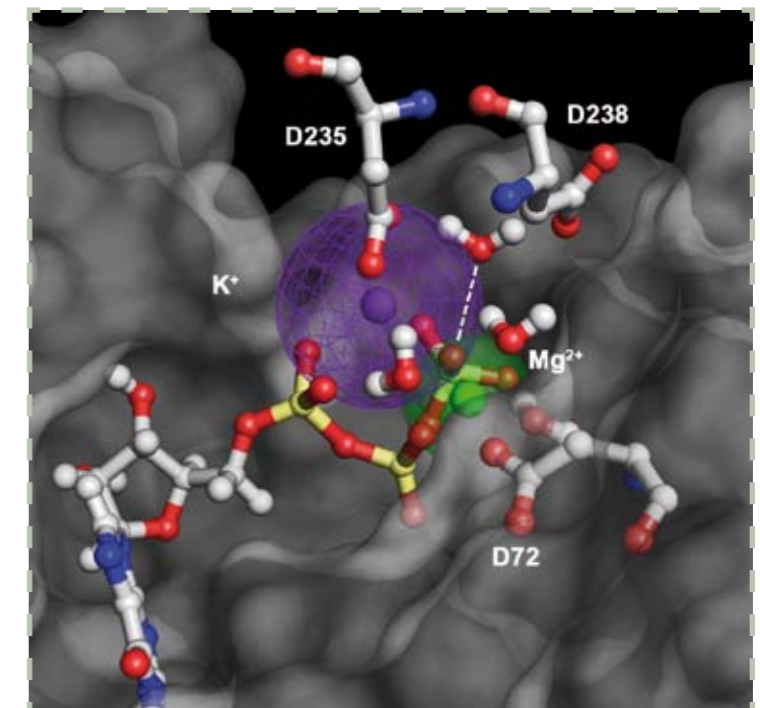
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Molecular Dynamics modelling of FtsZ GTPase activity. Scheme of the position of selected atoms surrounding GTP in the FtsZ dimer interface after 5 ns simulation in presence of potassium. A water molecule is located within the coordination sphere of potassium (purple) in a position compatible for hydrolysis of the GTP molecule (Mingorance et al., 2010. *Trends Microbiol* 18:348-356).



**Plant
Molecular
Genetics**

Research conducted by the Plant Molecular Genetics Department aims to uncover the signaling pathways underlying plant's ability to adapt to a variable environment and defensive responses against pathogenic diseases. Studies carried out by the different groups cover important aspects of Plant Biology such as stem cell function, root architecture, shoot branching, responses to day length duration and light quality, innate-immunity and defense responses to pathogens, as well as adaptive responses to nutrient shortage or heavy metals, since all these processes are known to be important determinants of biomass production and crop harvest yields. Biochemical, cell biology, genetic and genomic approaches are used to tackle the regulatory pathways underlying these developmental processes, in addition to the analysis of natural variation in these important responses or the role of targeted protein destabilization in their activation.



CARLOS ALONSO BLANCO
Genetic and Molecular Bases of the Naturally-Occurring
Variation of Plant Development

104

CARMEN CASTRESANA
Plant Immunity Strategies Against Microbial Pathogen Infection

106

PILAR CUBAS DOMÍNGUEZ
Genetic Analysis of Axillary Meristem Development

108

JUAN ANTONIO GARCÍA & CARMEN SIMÓN
Plant-Pathogen Interaction in Viral Infections

110

ANTONIO LEYVA
Molecular Mechanisms Underlying Root Architecture and Arsenic Phytoremediation

112

JOSÉ MIGUEL MARTÍNEZ-ZAPATER
Grapevine Reproductive Biology

114

JAVIER PAZ-ARES & VICENTE RUBIO
Control of Plant Responses to Phosphate Starvation

116

SALOMÉ PRAT
Hormonal Cross-Talk in Light Signalling and Day Length Control of Potato Tuber Formation

118

ENRIQUE ROJO
Intracellular Trafficking in Plants

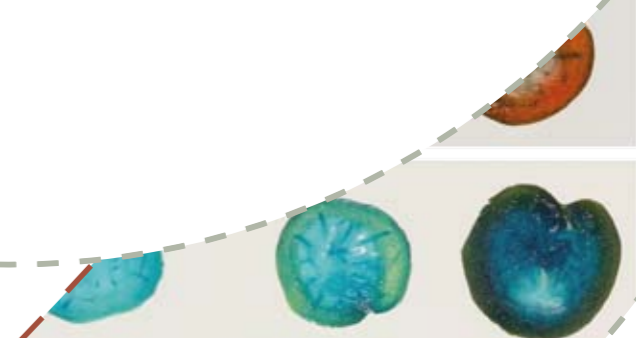
120

JOSÉ J. SÁNCHEZ-SERRANO
Wound Signalling in Plants

122

ROBERTO SOLANO
The Jasmonate Signalling Pathway in *Arabidopsis*

124



Plant Molecular Genetics

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Genetic and Molecular Bases of the Naturally-Occurring Variation of Plant Development

The main objective of our research is to understand the genetic and molecular mechanisms involved in plant adaptation.

We are dissecting the genetic variation in the model annual plant *Arabidopsis thaliana* in nature. Similar to many other plant species, individuals and populations of *A. thaliana* living in different geographical regions differ in many developmental traits that are presumed to reflect adaptations to different environments. To exploit this genetic variation for understanding plant adaptation, our group is currently focused on two specific objectives.

On the one hand, we are carrying out genetic analysis of the naturally-occurring variation for a key quantitative developmental trait such as the timing of flowering. We have developed several populations of recombinant inbred lines (RIL) and introgression lines (IL). We have analysed the relationship between the genetic basis of flowering initiation and the rate of vegetative growth in a new RIL population of 222 lines derived from the cross LerxFei-0 (Figure 1). This population has been used for QTL (quantitative trait locus) mapping analysis, which indicated 10 genomic regions that show two distinct patterns of pleiotropic effects on both traits (Méndez-Vigo et al., 2010).

On the other hand, we have developed a collection of wild genotypes of *A. thaliana* from the Iberian peninsula, which serve as a permanent experimental population on which to carry out genetic and environmental association analyses. As a first step in exploiting this population, we characterized 182 Iberian genotypes for their flowering response to vernalisation and have sequenced several flowering genes. We found new wild allelic series of some flowering genes by association mapping. In addition, analysis of the geographic and climatic distribution of gene polymorphisms is enabling us to identify alleles that might be involved in climatic adaptation.

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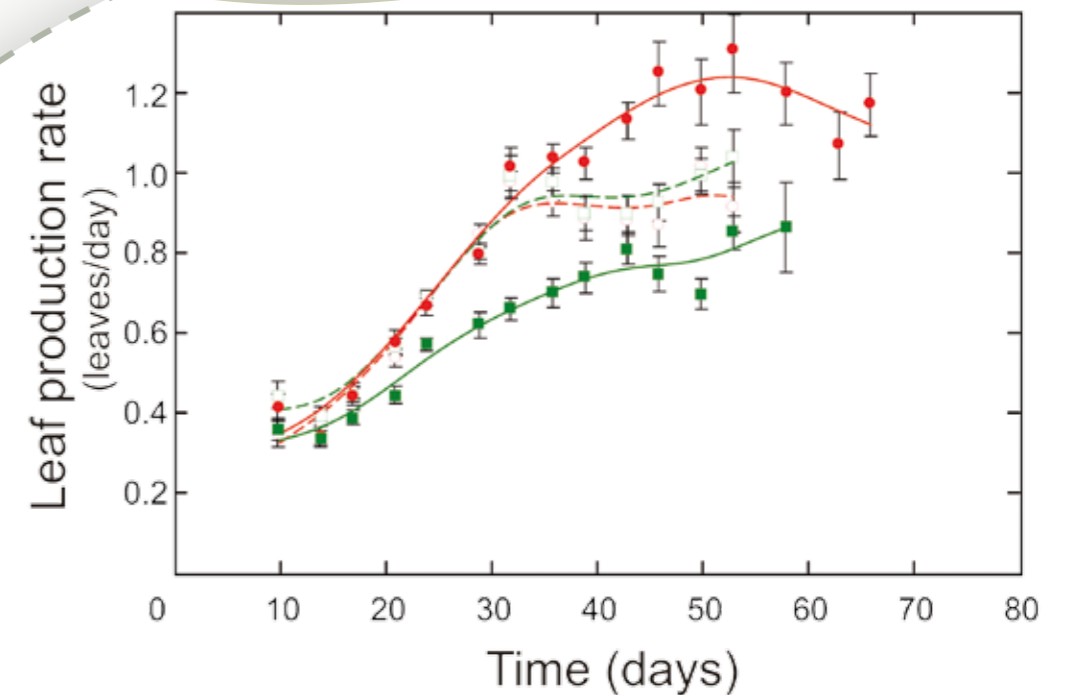
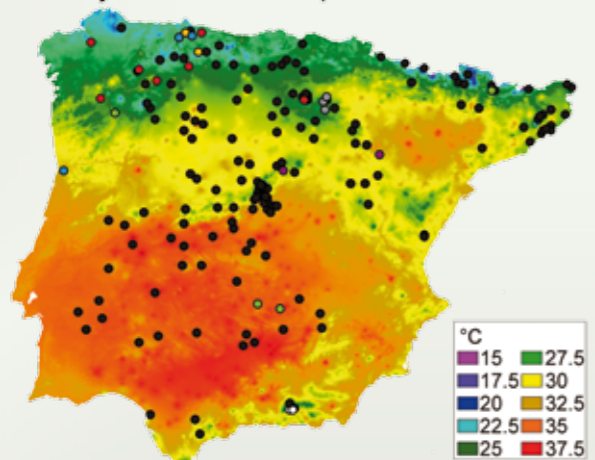
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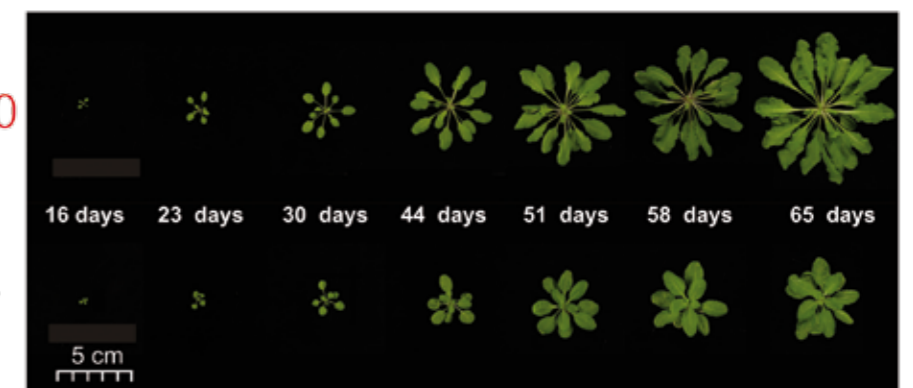


Geographic and climatic distribution of FRI truncations in the Iberian Peninsula. FRI functional alleles are shown as black dots, while FRI loss-of-function (truncation) alleles are shown in other different colors. The climatic map of the Iberian Peninsula corresponds to maximum July temperature.

July maximum temp:FRI truncations



Fei-0



Ler

Rate of vegetative growth of Ler, Fei-0 and their reciprocal hybrids.

Plant Molecular Genetics

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Plant Immunity Strategies Against Microbial Pathogen Infection

An ubiquitous response of plants to pathogen attack is the generation of active lipid derivatives, collectively known as oxylipins, whose importance as regulators of plant defence is being established.

Such compounds can be formed by enzymatic peroxidation of fatty acids, catalysed by the activities of 9- and 13-lipoxygenases and α -dioxygenases, or non-enzymatically in the presence of singlet oxygen and free radicals. The importance of the oxylipin pathway initiated by 13-lipoxygenases (13-LOX) and its main product, jasmonic acid (JA), in plant fertility and in controlling resistance to necrotrophic pathogens has been demonstrated. In addition, increasing experimental evidence indicates the participation of oxylipins produced by the 9-LOX and α -DOX pathways, as well as of non-enzymatically generated oxylipins in plant defence. Nevertheless, with the exception of JA, the signalling mechanisms by which distinct oxylipins exert their function remain poorly understood.

We aim to define the role of oxylipins in protecting plants against pathogen infection and to dissect the signalling pathways mediating their actions. During this period we undertook a genetic approach to investigate the signalling processes regulated by 9-HOT, a 9-LOX-derivative that induces production of callose deposits, initiation of oxidative stress and transcriptional changes of defence-related genes. A *lox1 lox5* mutant, which is deficient in 9-LOX activity, and the mutant *noxy22* (*non-responding to oxylipins22*), which is insensitive to 9-HOT, were used for this purpose. Map-based cloning positioned the *noxy22* mutation at the *ETO1* (ETHYLENE-OVERPRODUCER1) locus with constitutive ethylene (ET) production. As predicted from these results, we found that ET acts as a negative regulator of the 9-HOT signalling and, reciprocally, that 9-HOT interferes with the activation of the ethylene (ET) pathway.

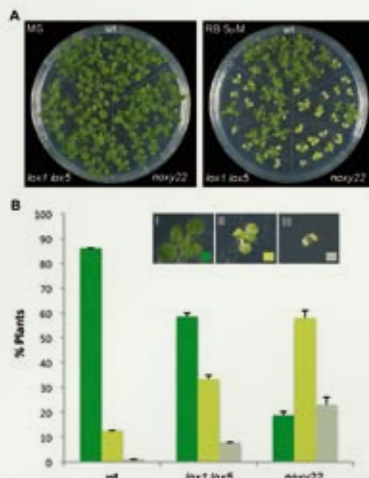
We found that the 9-HOT and ET pathways play a critical role in controlling oxidative stress and the response to the lipid

peroxidation-inducer singlet oxygen. Thus, the massive transcriptional changes seen in wild type plants in response to singlet oxygen were greatly affected in the mutants examined. Accordingly, *lox1 lox5* and *noxy22* displayed enhanced susceptibility to singlet oxygen (Figure 1). Further studies revealed the participation of the 9-LOX and ET in the defence of plants against *Pseudomonas* infection. Results showing enhanced susceptibility of *lox1 lox5* and *noxy22* to *Pseudomonas* attack and altered ROS homeostasis supported the role of the 9-LOX oxylipin pathway in controlling oxidative stress during plant defence against biotrophic bacteria, and the negative role of ET in the defence response against this type of pathogens.

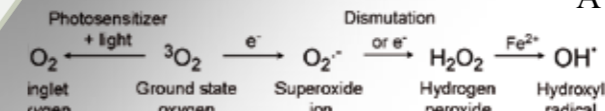
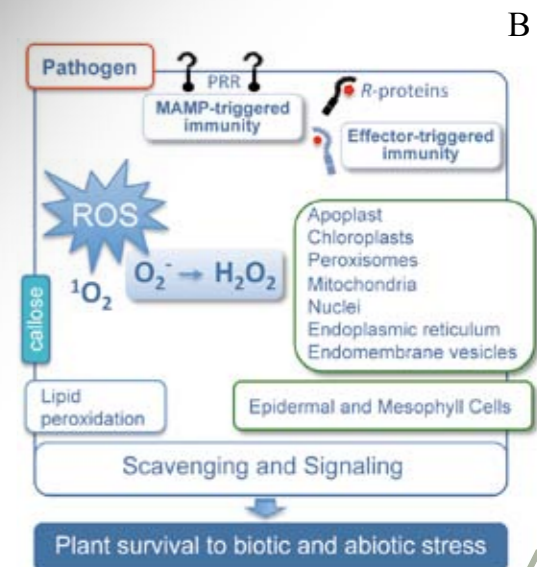
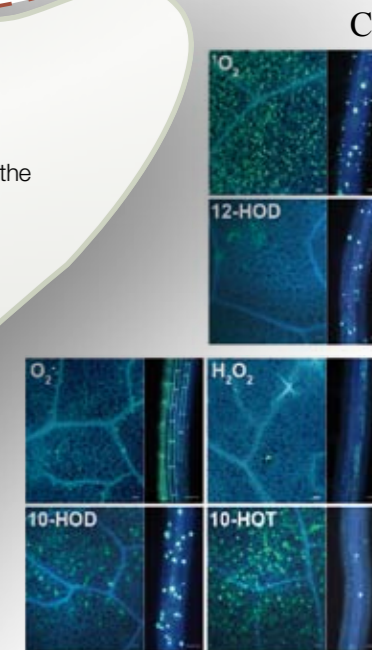
Given the participation of the 9-LOX oxylipin pathway in plant defence and in controlling the response to singlet oxygen (1O_2), we are investigating the role of this reactive molecule and of the 1O_2 -formed oxylipins in the immune strategies of plants against microbial infection. Singlet oxygen is produced as part of an oxidative burst that takes place after pathogen attack, in which oxygen is converted in distinct reactive oxygen species (ROS), such as the superoxide anion radical (O_2^-), the hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^*). Polyunsaturated fatty acids are a preferred target of 1O_2 at attack, and several of its oxidation products (non-enzymatically generated oxylipins) could act as secondary messengers to trigger defence responses. In support of this idea, we found that production of 1O_2 (triggered by Rose Bengal) and application of 1O_2 -formed hydroxy acids (12-HOT, 10-HOT, 10-HOD) induce a strong accumulation of callose, a marker of the plant response to pathogen attack, in leaves and roots of *Arabidopsis* (Figure 2). Moreover, we found that application of 12-HOT provoked a strong transcriptional response in which ~50% of genes are defence-related. Further studies are underway to define the role of singlet oxygen and of 1O_2 -formed oxylipins in plant defence.

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Analyses of phenotypic alterations in wild type, *lox1 lox5*, and *noxy22* plants grown in the presence of Rose Bengal, used as generator of singlet oxygen. A. Phenotype of seedlings grown during 15 days in MS medium (left) or in MS RB-containing medium (5 μ M) (right). B. Phenotypic alterations were scored in a three-point scale, designated as I, II and III, according to the severity of the symptoms. Bar graph indicates the percentage of seedlings showing the phenotypes observed after 15 days of growth in RB-containing medium. Sample means and standard errors from three independent experiments are shown.



**Plant
Molecular
Genetics**

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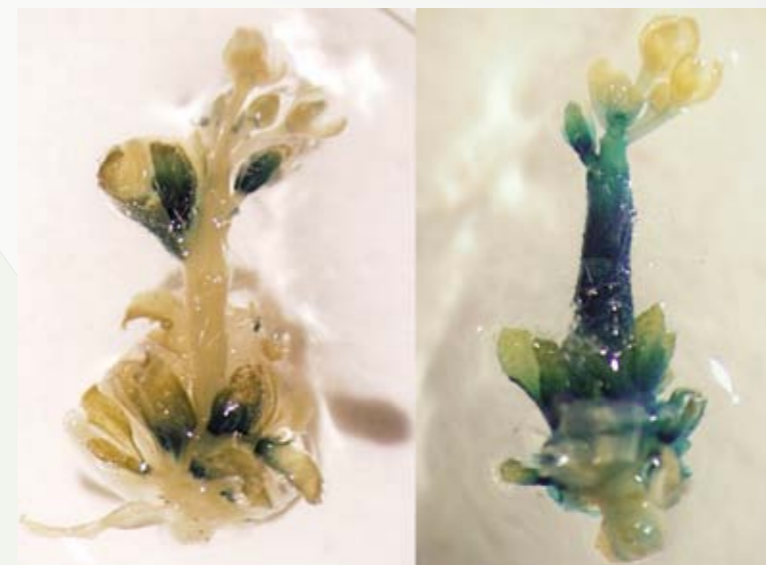
Genetic Analysis of Axillary Meristem Development

We are studying the genetic basis of the control of axillary bud development in the model system *Arabidopsis*, and in the crop species tomato and potato in which control of lateral shoot branching is of great agronomical interest.

We have characterised the *Arabidopsis* BRANCHED1 (*BRC1*) gene, which acts as a central switch of axillary bud development and outgrowth. We are now expanding our knowledge of the genetic networks involving *BRC1* in *Arabidopsis*. First, we identified and characterised two types of motifs conserved in the *BRC1*-like promoters, which act as transcriptional silencers of *BRC1*. One of these elements confers qualitative (spatial) regulatory information essential for driving *BRC1* expression in axillary buds; the others act as silencers, necessary to maintain low *BRC1* expression levels. We have identified two transcription factors that could bind to one of these elements and might be involved in the negative transcriptional regulation of *BRC1*. Second, we have begun to understand the protein-protein interactions involving *BRC1* and have isolated two factors that could modulate *BRC1* activity. In addition, we identified the *BRC1* protein domains mediating those interactions. Third, we compared the transcriptomic profiles of wild type and *brc1* axillary buds and identified genes that could be controlled directly by *BRC1*. We are systematically analysing the genetic control of branch suppression in response to shade in *Arabidopsis* and testing the working hypothesis that during the SAS, *BRC1* is upregulated in response to reduction in the R:FR ratio and its activity is responsible for the branch suppression response.

Solanaceae is a family that includes a large number of species in which the control of branch outgrowth is of great agronomical interest, and for which understanding the function of some of the key players will help optimise plant

architecture and yield. Our work shows that in tomato and potato, species with branching patterns divergent from those of *Arabidopsis*, two *BRC1*-like



Left: *Arabidopsis* transgenic plants carrying the promoter of *BRC1* fused to *b*-glucuronidase (*GUS*) display *GUS* activity in axillary buds. Right: similar plants carrying a mutated *BRC1* promoter display ectopic *GUS* activity.

paralogues, *BRC1a* and *BRC1b*, are coexpressed in axillary buds. Reverse genetic analyses confirmed that tomato *SIBRC1b* plays a role in the promotion of axillary bud arrest. In contrast, *SIBRC1a*, which encodes a divergent protein with a novel C-t domain, has a still unclear role in this process. Evolution rate studies indicate that whereas *BRC1b* has evolved under a strong purifying selection in the clade comprising *S. Lycopersicon*, other closely related wild tomato species and potato, *BRC1a* has evolved at a faster rate under positive selection.

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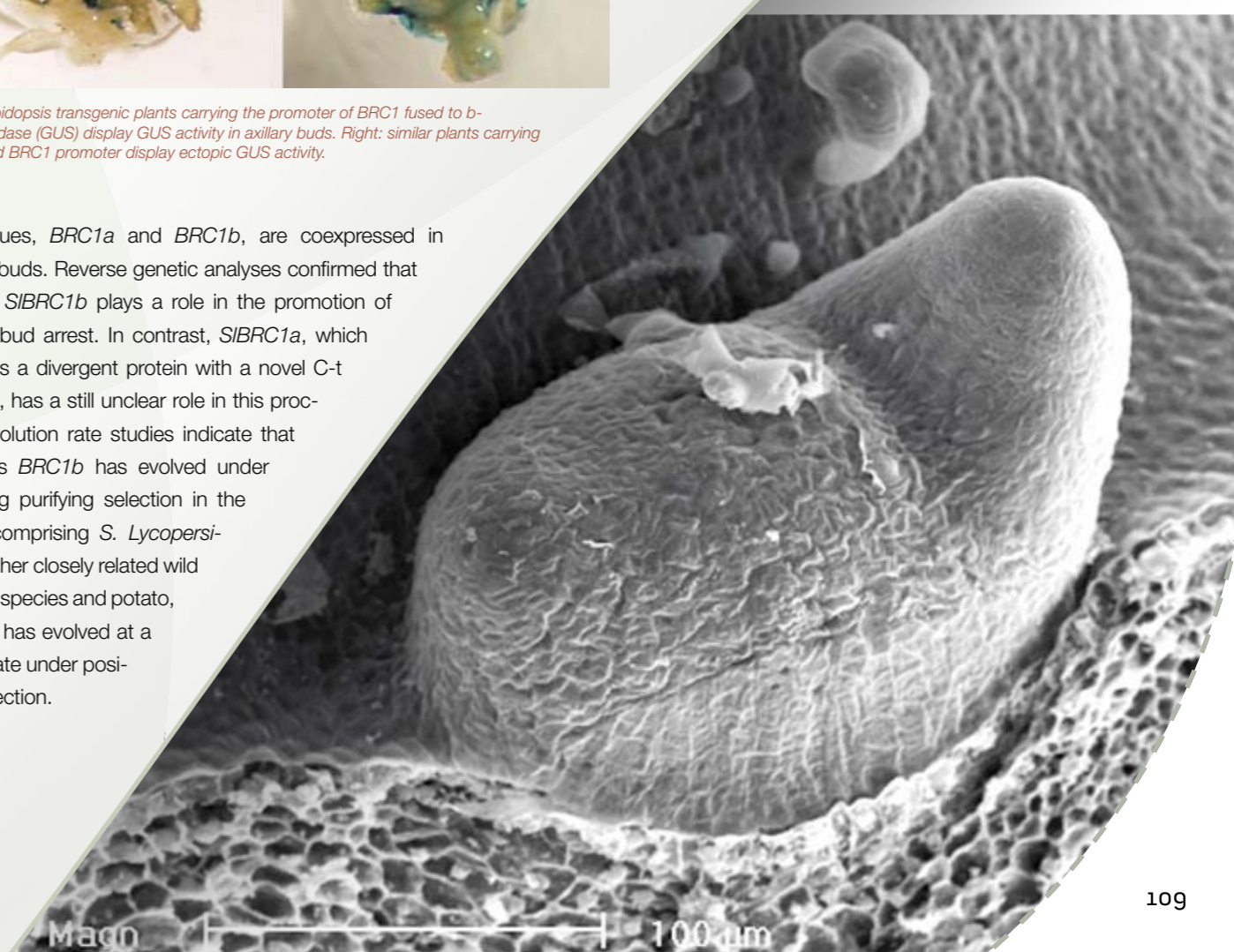
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Scanning Electron Microscopy image of a young tomato axillary bud.



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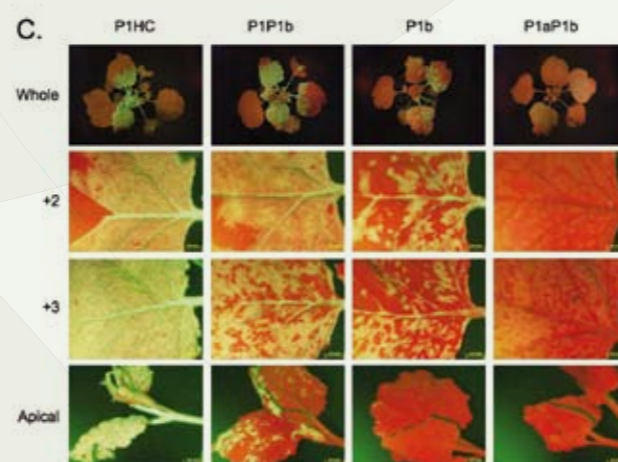
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**Plant-Pathogen Interaction
in Viral Infections**

Plant viruses depend largely on host factors to replicate in the cell and to propagate throughout the plant and between individual plants.



Infection of wild type PPV (P1HC) or chimeric PPV viruses in which P1HCpro has been replaced by CVYV P1b alone (P1b) or preceded by PPV P1 (P1P1b) or CVYV P1a (P1aP1b). All viruses are tagged with GFP. A western blot of the infected tissues is shown at the left.

Plants in turn have developed antiviral defence mechanisms that must be counteracted by viral factors. These factors appear to be preferred targets for alternative plant defences. In our laboratory, we try to understand this complex interplay, mainly in the infection of the potyvirus *Plum pox virus* (PPV), the causal agent of sharka, a damaging disease of *Prunus* trees. We are especially interested in defence responses related to RNA silencing and its viral suppressors.

HCPPro is the typical silencing suppressor of potyviruses, but other silencing suppressors of *Potyviridae* family members have recently been discovered. We demonstrated that serine proteinase P1b of the ipomovirus *Cucumber vein yellowing virus* (CVYV) suppresses silencing by sequestering siRNA, and can replace HCPPro functionally in PPV infection in a host-specific manner. Our results demonstrate that although potyviruses can exploit different sources of anti-silencing activity, their own silencing suppressors can con-

tribute to defining the specific host range of the virus. We also showed that single amino acid changes at the N-terminal region of the capsid protein (CP) control specific PPV adaptation to *Prunus persica* and *Nicotiana* species. Our findings suggest that species-specific interactions of the CP N-terminal region with host factors have an important role in viral long distance movement, and that an unknown resistance mechanism interferes with these interactions in *Nicotiana* species. We showed that the N-terminal region of PPV CP is O-GlcNAcylated and phosphorylated. An O-GlcNAcylation-deficient PPV mutant infects *Nicotiana clevelandii* and *P. persica* with no apparent differences compared to wild type PPV. In contrast, PPV accumulation is significantly lower in *Arabidopsis thaliana* Col-0 plants infected with the mutant virus, concurring with a fine-tuning effect of CP O-GlcNAcylation on PPV infection that facilitates its adaptation to different hosts and environmental conditions. We are interested in applying the information obtained in our research to design control strategies for sharka disease. We thus used RACE assays of viral RNA fragment accumulation in infected plants as well as deep sequencing of viral siRNA to design 20 PPV-specific amiRNA constructs. Cleavage activity on sensors and a high level of antiviral protection in agroinfiltration systems was shown for some of the amiRNA, which will be transferred to other PPV plant hosts. Finally, another target of interest is development of PPV-based plant expression vectors. To broaden the range of plants susceptible to PPV-based vectors, we developed an infectious cDNA clone of a PPV isolate of strain C, the only one infecting cherry trees in nature. Biological features of this cloned isolate are being analysed.

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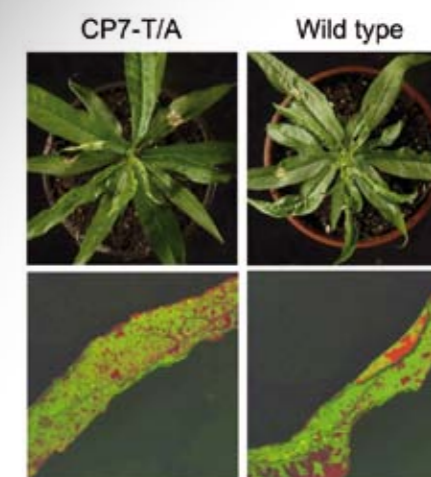
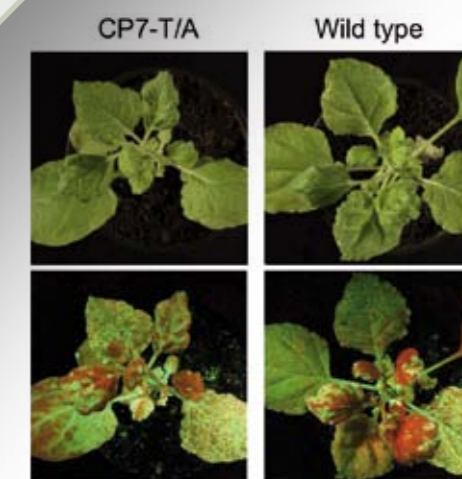
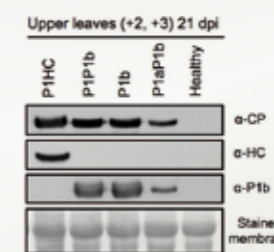
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Infection of the O-GlcNAcylation-deficient mutant CP7-T/A in *Nicotiana benthamiana* and *Prunus persica*. The mutant and the wild type PPV are tagged with GFP.

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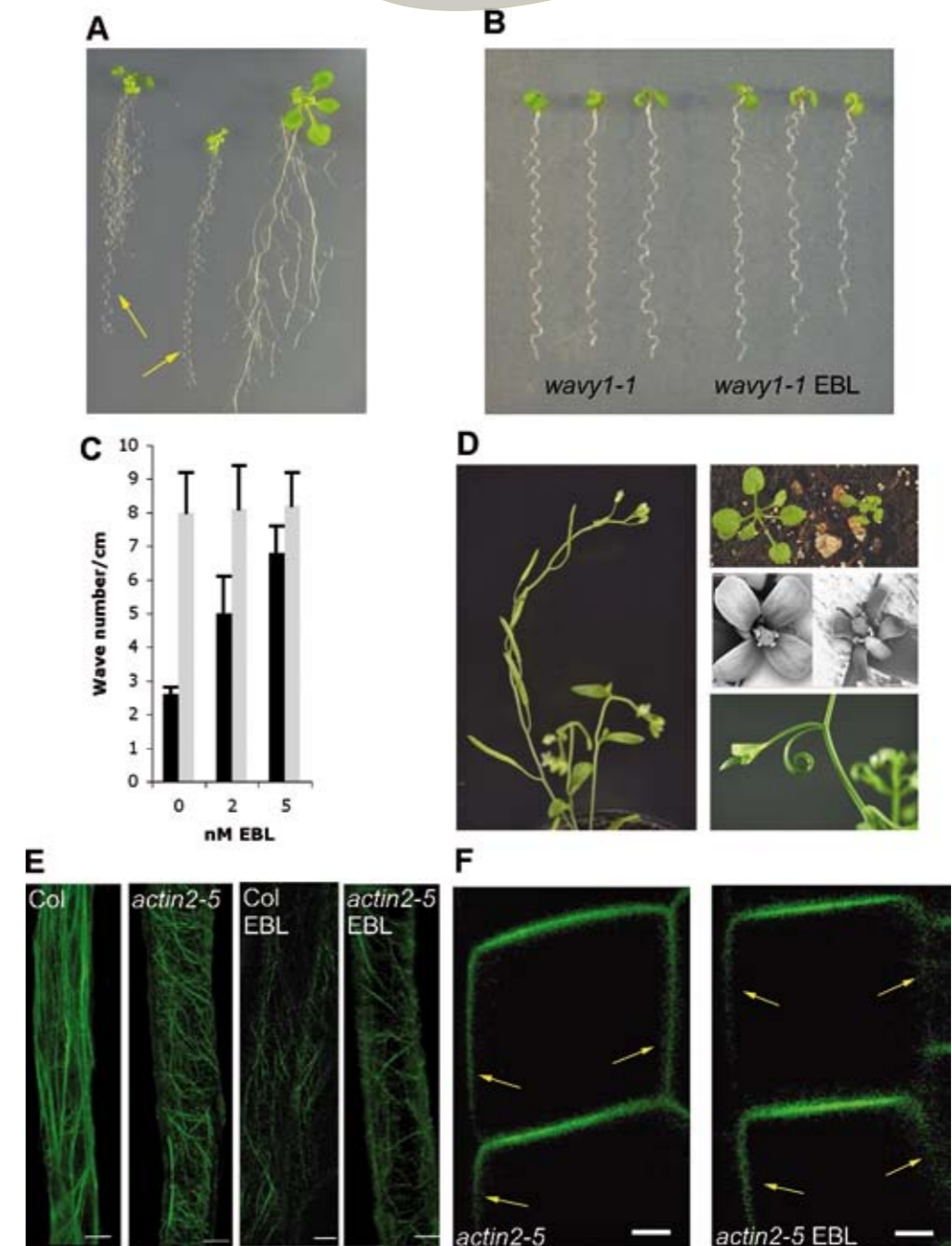
Molecular Mechanisms Underlying Root Architecture and Arsenic Phytoremediation

We are developing two research lines in the laboratory.

Our first aim is to study the molecular mechanisms underlying arsenic perception and the second, to study the mechanisms involved in root architecture. For the latter, we performed a mutant screening in *Arabidopsis* to identify mutants altered in the spatial distribution of roots. From this screening, we identified several mutants altered in root architecture, including *actin2-4*, a new mutant allele of the *ACTIN2* gene. This mutant shows an increase in actin dynamics, leading to the enhancement of tropic responses and auxin transcriptional responsiveness, mimicking the auxin/brassinosteroid synergistic response. In the first research line, mechanisms underlying arsenic perception, we made two key observations. Using a transgenic plant that expresses the Pi transporter promoter fused to the luciferase gene, we identified a set of genes inducible by Pi starvation that are repressed by As(V) with extraordinary speed (30 min), whereas repression by Pi takes 36 hours. The use of the transgenic line PHT1;1:LUC opens the possibility to identify mutants altered in the kinetics of repression by As(V), being a new approach for the characterization of the signalling pathway of As(V) and its possible cross-talk with that of Pi. Moreover, in the context of this project we have performed an analysis of the natural variability of tolerance to As(V) in a large collection of *Arabidopsis* ecotypes. In this analysis we identify a QTL that we are currently cloning.

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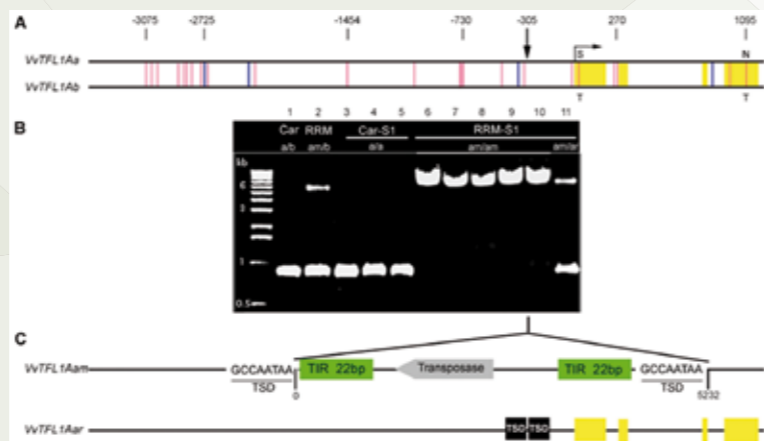
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Grapevine Reproductive Biology

Starting in March 2009 most of our group moved to the recently created Instituto de Ciencias de la Vid y del Vino (Grapevine and Wine Research Institute) in Logroño.



VvTFL1A alleles in cultivar Cariñena and RRM somatic variant. A. Wild type alleles. B. Mutant allele with transposon insertion. C. Revertant allele showing the target site duplication.

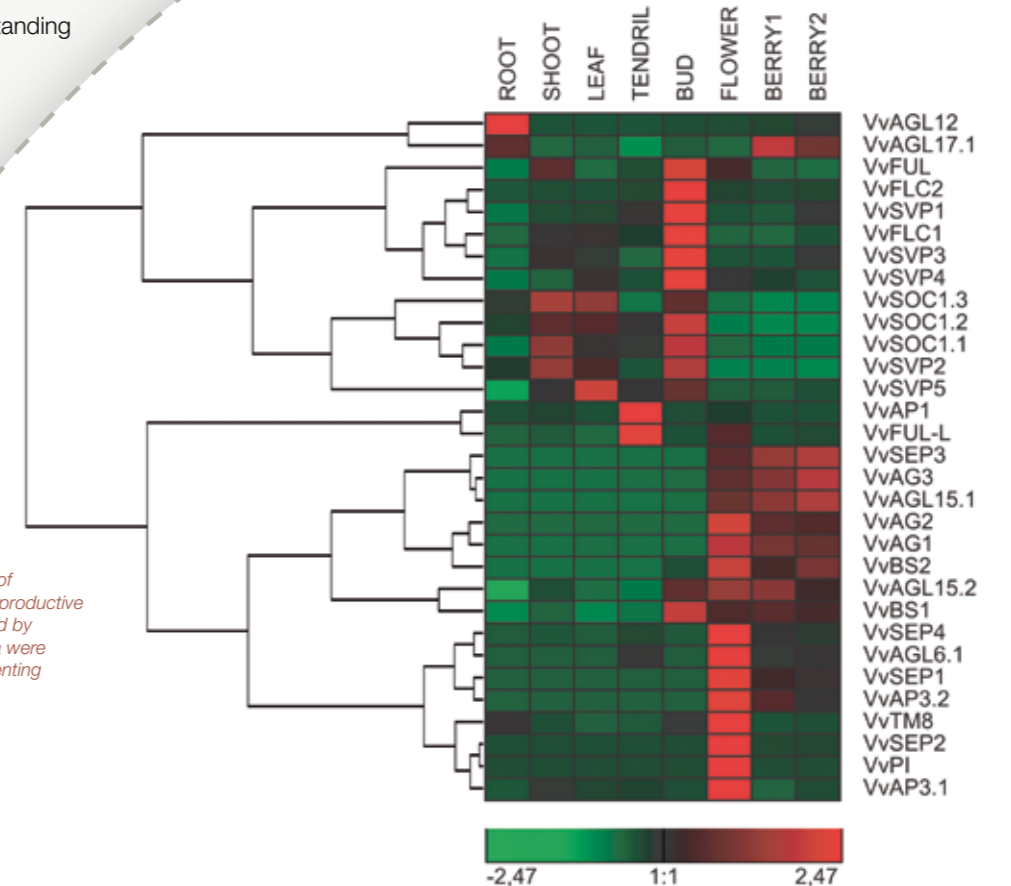
This Institute derives from a joint agreement between the Agencia Estatal CSIC, the University of La Rioja and the Government of La Rioja. However, part of the research group is still maintained at the CNB till the running projects reach to their end.

Our research interests focus on the understanding of the genetic and molecular mechanisms regulating reproductive development in grapevine and their contribution to final production and quality of grapes and wines. In the last two years our research activity has been centred on the characterization of grapevine natural genetic variation for reproductive traits and its use to identify some of the underlying loci and genes. With this purpose we are generating core collections of grapevine cultivars, collections of somatic variants and F1 hybrid progenies derived from crosses between different cultivars. The genetic analyses of these materials have allowed us the identification of QTLs for relevant reproductive traits such fertility, cluster structure, berry shape and size or seedlessness. In addition, the molecular analyses of somatic variants such as *Reiterated Reproductive Meristems* (RRM)

in cultivar Cariñena has allowed the identification of a putatively active transposable element of the *Hatvine1* family as responsible for over-expression of the *VvTFL1A* in a natural activation tagging event. (Figure 1) Over-expression of *VvTFL1A* seems to be directly related to the RRM phenotype.

In addition we are involved in the genomic analyses of reproductive development in grapevine, performing both a manual annotation of gene families regulating the process as well as its transcriptional analyses. Annotation of the MIKCC-type MADS box gene family in grapevine has shown the existence of at least 38 genes clustered in 13 subfamilies. Expression profiles of MIKCC-type genes in vegetative and reproductive organs as well as during flower, tendril and fruit development show conserved expression domains for specific subfamilies but also reflect characteristic features of grapevine development. Expression analyses in latent buds and during flower development reveal common features previously described in other plant systems as well as possible new roles for members of some subfamilies during flowering transition (Figure 2). The analysis of MIKCC-type genes in grapevine helps understanding the origin of gene diversification within each subfamily providing the basis for functional analyses of these MADS box genes in grapevine development.

Cluster analysis of gene expression profiles of grapevine MIKCC genes in vegetative and reproductive organs. Expression analyses were performed by qRT-PCR, and relative gene expression data were gene-wise normalized. Color scales, representing signal values, are shown at bottom.



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Control of Plant Responses to Phosphate Starvation

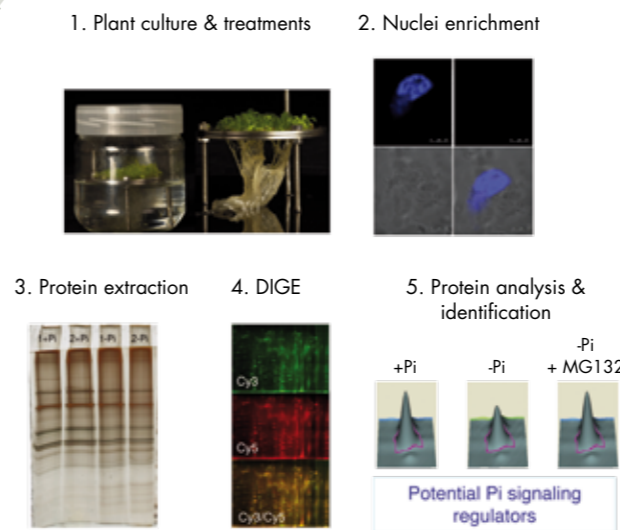
Plant responses to phosphate (Pi) starvation represent an emblematic system for studies on regulation of gene activity.

In plants, these responses involve both biochemical and developmental changes that improve Pi acquisition and recycling, and protect against the stress of Pi starvation. The induction of Pi starvation responses requires a sophisticated regulatory system that integrates information on external and internal plant Pi concentration, and on other nutrient content. Our aim is to contribute to the dissection of this regulatory system, which operates mainly at the transcriptional level but also involves post-transcriptional control.

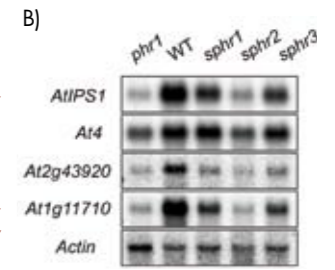
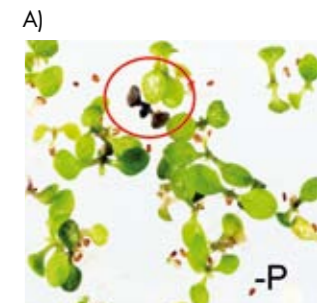
Regarding transcriptional control, we have further demonstrated the key role of the transcription factor phosphate starvation response regulator1 (PHR1) and its binding site (P1BS) as a master trans-cis integrator module in the control of Pi stress responses. We also initiated large-scale phylogenomic footprinting to identify all cis-motifs involved in Pi starvation responsiveness. To identify regulatory mechanisms that act on PHR1, we carried out a screening for *phr1* suppressors, leading to the isolation of three mutants that partially suppress *phr1* mutant phenotypes. Cloning of one of the corresponding mutant genes revealed it corresponds to ALIX, a gene conserved from yeast to mammals. ALIX regulates ubiquitination and subsequent endocytosis of plasma membrane receptors, essential for signal propagation from these receptors (Fig. 1). Our hypothesis is that ALIX regulates Pi status signalling by direct interaction with a yet unknown Pi sensor.

Concerning post-transcriptional control, we started a program to study the biochemical and physiological functions of the ubiquitin (Ub) regulatory pathway in the control of plant responses to Pi starvation. We identified a small subfamily of E3 Ub ligases (PCE1-4) that control Pi starvation responses, as indicated by characterization of *Arabidopsis pce* null mutants. PCE proteins are nuclear-localized and its abundance is reduced by Pi addition, indicating a potential role as repressors of Pi signalling. Using a yeast two-hybrid approach, we identified a number of potential targets of PCE1 activity, including known transcription factors involved in control of plant growth and development. The

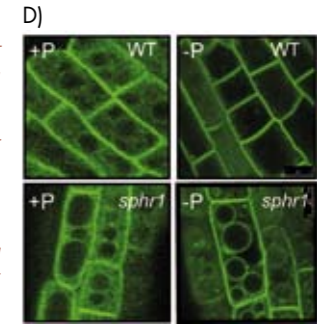
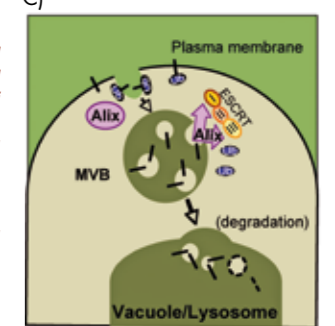
biological relevance of these interactions is currently being addressed. As a complementary approach, we are analyzing variation in the abundance of nuclear proteins that depend on Pi supply and Ub-proteasome activity. We have focused on the nuclear proteome, since nuclear proteins probably have regulatory activities. Using 2-D fluorescence difference gel electrophoresis (DIGE) techniques (available at the CNB Proteomics Facility), we have identified four proteins with a potential regulatory role in Pi signalling whose characterization is ongoing (Fig. 2).



Analysis of changes in the nuclear proteome of *Arabidopsis* in response to variations in Pi supply. Study of the effect of ubiquitination and proteasomal degradation. To identify potential substrates of the Ub-proteasome pathway with a regulatory role in Pi signalling, we are analyzing variation in the abundance of nuclear proteins that depend on Pi supply. We have established both nucleus isolation and nuclear protein extraction protocols for *Arabidopsis* plants grown in hydroponic media under different Pi conditions (steps 1-3). Nuclear proteome separation, visualisation and protein abundance quantification are carried out by DIGE techniques (step 4). By adding proteasome inhibitors (e.g., MG132) in the culture media at specific time points, we can determine the effect of ubiquitination and proteasomal degradation in the abundance of Pi-responsive proteins. The nature of each protein identified, using mass spectrometry techniques, provides hints about their potential regulatory function in Pi signalling (step 5). Functional characterization of the most interesting proteins will allow us to test their involvement in Pi homeostasis.



Identification and characterization of suppressors of the *phr1* mutation. The fact that *phr1* mutants accumulate reduced levels of anthocyanins in response to Pi starvation compared to WT plants (A) allowed us to carry out mutant screening to identify mutants that suppress the effect of the *phr1* mutation in this response (B). Suppression of the *phr1* phenotype in the mutant lines identified (*sphr*) also spanned to the expression of Pi starvation-responsive genes (B). *SPHR1*, isolated using positional cloning strategy, is an orthologue of ALIX, a gene conserved in eukaryotes that regulates trafficking of ubiquitinated proteins from plasma membrane to lysosomes (vacuoles in plants) through endosomes and multivesicular bodies (MVB). ALIX interacts with members of the ESCRT complexes, triggering degradation of specific plasma membrane proteins, which helps to control their abundance and function (C). In accordance, *sphr1* mutants display defects in endocytic trafficking of high affinity Pi transporters fused to the green fluorescent protein (GFP) compared to wild-type (WT) plants (D).



Plant Molecular Genetics

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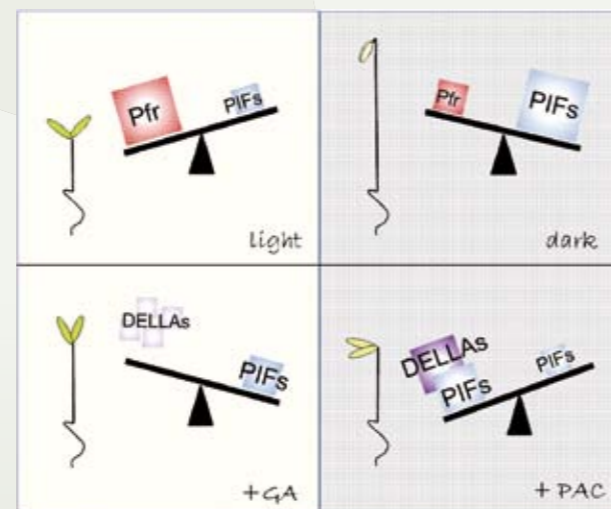
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Hormonal Cross-Talk in Light Signalling and Day Length Control of Potato Tuber Formation

Light is a crucial environmental signal to plants since it provides energy for photosynthesis and in addition serves as an informational cue of the ambient in which the plant grows.

Important light-regulated responses are the de-etiolation of seedlings after seed germination and the response to shade in adult plants, aimed to cope with the competition imposed by other plants. These two developmental responses which are triggered by light/darkness or by changes in the R/FR light ratio are to a large extent regulated by the transcription factor PIF4 (PHYTOCHROME INTERACTING FACTOR 4), which is destabilized by the PHYB photoreceptor in the light. Work in our group aims to identify which signaling cascades govern etiolated seedling development in the dark and how these cascades are affected by light, with a particular focus in the mechanisms involved in integration of the light signal with the own running developmental programs of the plant. The plant hormones gibberellins (GA) and brassinosteroids (BRs) play a central role in transducing the light signal as judged from the dark de-etiolated phenotype of mutants with a block in



PIF4/PIF5 integration of light and GA-signals. PIFs accumulate in darkness and promote hypocotyl elongation. In the light, the photoreceptor phyB marks these factors for degradation and represses hypocotyl growth. In the absence of GAs (+PAC), DELLAs bind to PIFs and block their DNA binding ability. GAs destabilize these repressors and lead to elongation of the hypocotyl.

the synthesis or response to these hormones. As for GA mutants, this phenotype is caused by the stabilization of the DELLAs, a family of nuclear proteins that repress GA-regulated gene expression and are rapidly destabilized in the presence of GAs. Evidence provided by our group showed that DELLAs bind the bHLH DNA recognition domain of the PIF factors and block DNA binding ability of these transcriptional regulators. PIF4 and its close homologue PIF5 regulate the expression of several genes driving cell elongation, by recognizing a G-box element in their promoters, the growth restraint imposed by DELLAs hence being mediated through a block in PIF4/PIF5-mediated activation of these genes.

Regarding BR-deficient/-response mutants an important finding was the observation that these seedlings are insensitive to GAs. This impaired response, however, does not correlate with an increased stability of DELLAs, suggesting that a regulatory step downstream of these repressors mediates GA-/BR- cross-talk. BRs were actually found to stabilize the PIF4/PIF5 factors in the light, these results pointing to a master regulatory role of these transcriptional regulators in the control of genes with a role in cell elongation, these TFs integrating not only light- (by PHYB-mediated destabilization) and GA- signals (by inactive complex formation with DELLAs) but playing a role also in BR signaling, thus serving as a molecular link between the signaling cascades controlling plant growth and elongation and the exterior.

A second important line of research in our group is day length control of storage organ formation in potato. It is well established that day length duration is perceived in the leaves, a tuberization signal known as tuberigen being synthesized in these organs and transported to the underground organs of the plant. Our work has demonstrated that this mobile signal is encoded by an FT-like gene (StSP6A), an additional member of the FT-like gene family (StSP3D/SFT) controlling floral transition independent of day length.

Two different FT paralogs control day neutral flowering and SD-dependent tuber formation in potato andigena species. The potato StSP6A gene encodes the mobile tuberigen signal. CONSTANS represses expression of this gene in LDs but promotes its activation in SDs, through an autorelay mechanism. The StSP3D plays a role in floral promotion and is induced in response to environmental cues other than day length.

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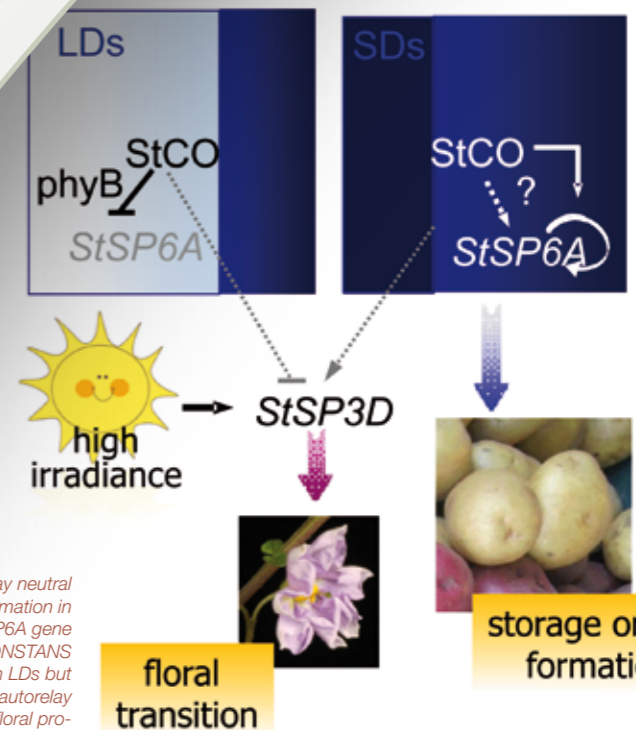
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PATENT

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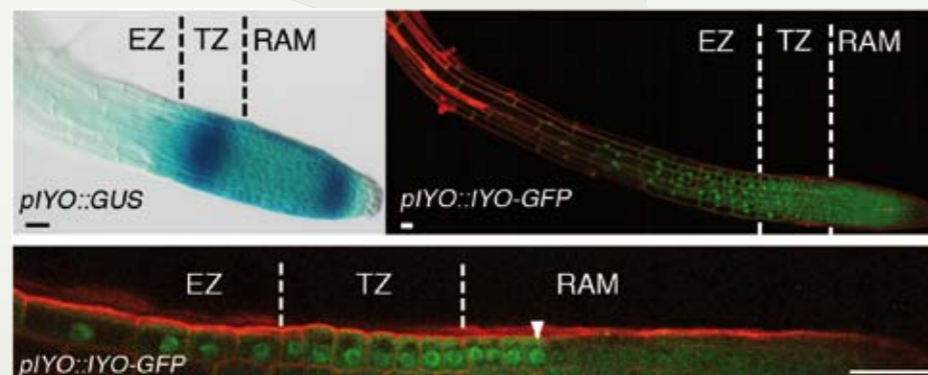
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Intracellular Trafficking in Plants

Recent work in our group has been focused in two separate topics.

First, we have continued the genetic dissection of storage protein trafficking to the vacuole in plants, through isolation of the *mtv* mutants impaired in this transport process. The first *MTV* gene isolated was isolated was *VT112*, a SNARE protein that forms a complex at the TGN required for transport of storage proteins, but not of other vacuolar cargo (Sanmartín et al., 2007). Other components of this pathway subsequently identified were an SM protein that positively regulates the *VT112* SNARE complex (Zouhar et al., 2009) and the sorting receptors for storage proteins (Zouhar et al., 20010). We are currently characterising other *MTV* proteins, such as *MTV8*, which contains a lipid-binding ENTH domain and is involved both in trafficking of storage proteins and in regulating plant senescence (Fig. 1). Several of the genes isolated are co-expressed and are activated during the massive deposition of storage proteins that occurs in maturing-seed embryos, suggesting that they are induced by a common mechanism to achieve full transport capacity in those cells. We have initiated a project to identify the cis regulatory elements and trans-acting factors that activate their transcription during seed maturation. The goal is to develop tools to activate in vegetative tissues the expression *en bloc* of this (limiting) transport machinery and determine whether storage capacity is altered.



Premature senescence in an *mtv8* mutant.

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A second topic in the lab was initiated through isolation of the *miniyo-1* (*iy0-1*) mutant, which has delayed organogenesis in the shoot apical meristem. Functional characterisation of *MINIYO* revealed that it is a necessary and sufficient factor for initiating all events of differentiation in *Arabidopsis*. Moreover, our results suggest that the targeted nuclear accumulation of *IYO* in transition cells (Fig. 2) functions as a transcriptional switch for this fate transition. We are now studying how endogenous and environmental signals regulate *IYO* expression and subcellular localization to control the onset of cell differentiation and, consequently, regulate plant growth and development.



IYO is expressed in meristems and the protein is transferred to the nucleus in transition cells, initiating differentiation. RAM: root apical meristem; TZ: transition zone; EZ: elongation zone.

**Plant
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Wound Signalling in Plants

Our research focuses primarily on wound signalling in plants, with *Arabidopsis thaliana*, tomato and potato as model systems.

Upon wounding, defence-related gene expression is induced at both damaged and distal tissues well apart from the wound site. The plant hormone jasmonic acid (JA) plays a pivotal role in the complex pathway that triggers activation of wound-responsive genes.

Our laboratory studies the role of JA and related compounds (oxylipins) in plant defence responses. We determined the localisation of various biosynthetic enzymes and studied possible interactions among them that could regulate oxylipin synthesis (Farmaki et al., 2007). Using transgenic overexpression and co-suppression approaches, we modified JA and oxylipin biosynthesis in transgenic potato and tomato plants, and studied the consequences on defence gene activation. Allene oxide synthase, which catalyses the first committed step in JA synthesis, is an important target of our research to elucidate a role for the isoforms found in potato and tomato. Overexpression of w-3 fatty acid desaturases in tomato results in production of higher levels of 3-hexenal, a potent antimicrobial oxylipin and a major component of the aroma of tomato fruit (Dominguez et al., 2010).

Reversible protein phosphorylation is a common molecular mechanism in the regulation of signal transduction pathways. We established that a regulatory phosphorylation switch controls the spatial and temporal pattern of wound-induced gene expression. In this switch, dephosphorylation of target protein(s) is essential for JA-dependent gene activation. Pharmacological studies indicate that PP2A and/or PP4 are the most likely candidates for dephosphorylating target effector proteins.

We have undertaken a reverse genetic approach to identify loss-of-function mutants in PP2A catalytic subunits (PP2Ac). These mutants will help to elucidate the role of

specific PP2A in the regulation of signal transduction networks in *Arabidopsis*. The *Arabidopsis* genome encodes five *PP2Ac* genes. Homozygous knockout mutant lines have been generated with T-DNA insertions in all *PP2Ac* genes. T-DNA-mediated disruption of the *PP2Ac-2* gene leads to ABA hypersensitivity. *PP2Ac-2* thus appears to be a specific negative regulator of ABA signal transduction in *Arabidopsis* (Pernas et al., 2007). However, all other single *PP2Ac* mutants show no obvious phenotypes, suggesting that the encoded proteins might play largely redundant roles, consistent with their high degree of similarity.

Double mutants have also been generated for each gene pair. Thorough characterisation of these mutants is revealing *PP2A* involvement in the regulation of developmental pathways and in responses to environmental stress. Identification and characterisation of target proteins that are specifically dephosphorylated by *PP2A* in these signalling pathways is one main goal in our future work.

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Phenotypes of wild type, single and enhanced mutant plants. (a) Genotypes are indicated for adult plants (top) and their corresponding rosette stage (bottom). (b) Wild type (left) and *pp2ac-3 pp2ac-4* double mutant (right) plants. Plants were grown on MS plates with 1% sucrose for 15 days. Detailed of *pp2ac-3 pp2ac-4* double mutant plantlets. Scale bar = 100 μ m.

Histochemical localisation of *GUS* activity in 13-HPL promoter lines. Tomato fruits at three developmental stages (mature green, orange and ripe red) were cut transversely and stained for *GUS* activity. WT: fruits from non-transformed *MicroTom* plants; Pro35S: fruits from the constitutive *CaMV 35S* promoter; PromHPL:*GUS*: fruit from a 2-kb HPL promoter transgenic line.



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The Jasmonate Signalling Pathway in *Arabidopsis*

We are interested in understanding how plants are able to perceive changes in their environment and integrate stress signals with their internal developmental programs to induce adaptive responses and survive in nature.

This integration depends on complex signalling networks that regulate the genetic reprogramming of the cell. The main focus of my lab is to understand one of the pathways involved in this network, the jasmonate (JA) signalling pathway, in *Arabidopsis thaliana*. Jasmonates are fatty acid-derived signalling molecules essential for plant survival in nature, since they are important activators of stress responses and developmental programs. We aim to identify the components of this pathway and determine how they explain jasmonate pathway interactions with other pathways in the network. We are also particularly interested in the molecular mechanisms underlying JA-mediated activation of plant responses to necrotrophic pathogens. This type of defence requires the concerted cooperation of at least two phytohormonal signalling pathways: JA and ethylene (ET). Since the plant uses these two hormones to signal many other developmental and stress responses, ET/JA crosstalk constitutes a unique (simple) system to study the regulation of signalling networks that allow the plant to discriminate between different stresses (e.g., pathogens and wounding) and select the correct set of responses to each.

To understand these biological questions, we are using genomic, genetic, biochemical and molecular tools, following two approaches:

1. Dissection of the JA signalling pathway in *Arabidopsis*. We have discovered several components of this pathway:

- The transcription factors (TFs) ERF1 and AtMYC2/JIN1, which regulate expression of two subsets of JA/ET-related effector genes (Lorenzo et al., 2003; Lorenzo et al., 2004). The balance of activation of these two TF helps determine the type of response activated by the plant to a specific stress (pathogens or wounding; Lorenzo and Solano, 2005).

- SGT1b/JAI4, a regulator of SCF (Skip-Cullin-Fbox) E3 ubiquitin ligase complexes, including the JA-signalling component SCF^{COI1} (Lorenzo and Solano, 2005; Feys et al., 1994; Xie et al., 1998; Xu et al., 2002; Devoto et al., 2002; Feng et al., 2003).

- The JAZ family of nuclear repressors that regulate TF activity (i.e., AtMYC2) and are targeted by SCF^{COI1} for degradation by the 26S proteasome (Chini et al., 2007). Discovery of the JAZ family of repressors linked the previous steps in the pathway (SCF^{COI1} and the TFs) and facilitated an integrated view of the core JA signalling module composed by SCF^{COI1}-JAZs-MYC2. It also evidenced the similarity between the JA and auxin pathways. Based on the crystal structure of the auxin receptor (F-box TIR1; closely related to COI1), we hypothesised that COI1 might also be the JA receptor. A combination of genetic and biochemical analyses supported this hypothesis (Fonseca et al., 2009). Using a screen for bioactive jasmonates, we found that the majoritary form of the hormone [(-)-JA-L-Ile] was inactive as a ligand of COI1, and discovered the real endogenous bioactive form of the hormone, (+)-7-iso-JA-L-Ile.

- NINJA, novel interactor of JAZ, is an adaptor protein that connects JAZ repressors to the general corepressor TOPLESS (Pauwels et al., 2010).

2. Dissection of the interaction between the oomycete *Pythium irregulare* and the plant *Arabidopsis thaliana*. We characterised the infection process and the hormone pathways involved in plant defence. We identified the genes responsible for plant resistance to this oomycete, and analysed the contribution of each of the "classical" defence hormones (JA, ET and SA) to the activation of this defence gene set. Finally, we found that abscisic acid (ABA) is essential for the activation of defences against *P. irregulare* and found that it precedes JA biosynthesis in response to the oomycete (Adie et al., 2007).

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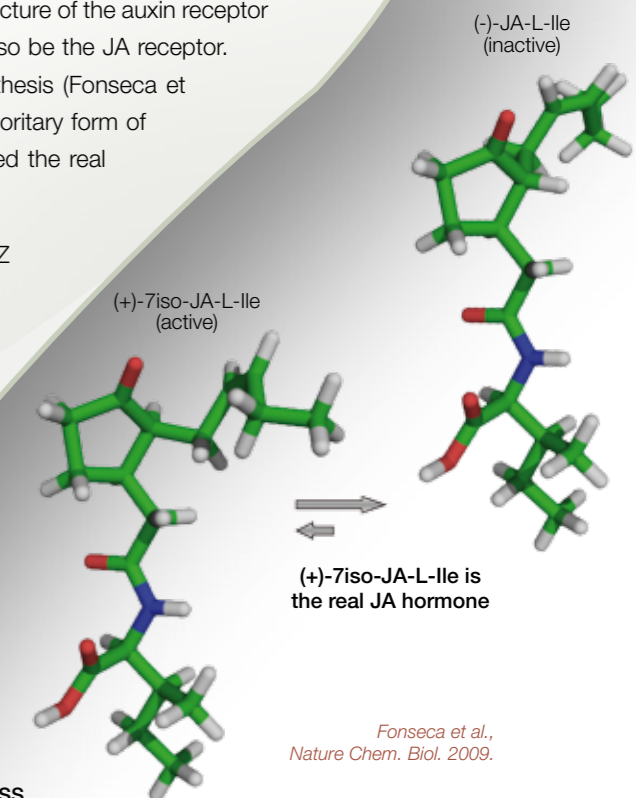
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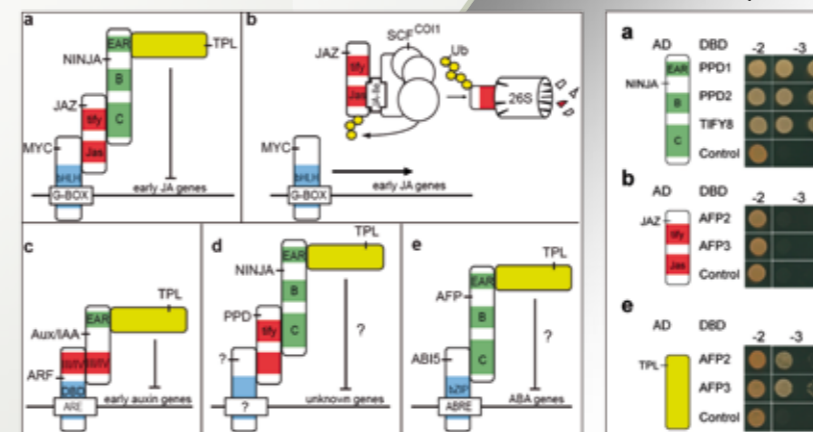
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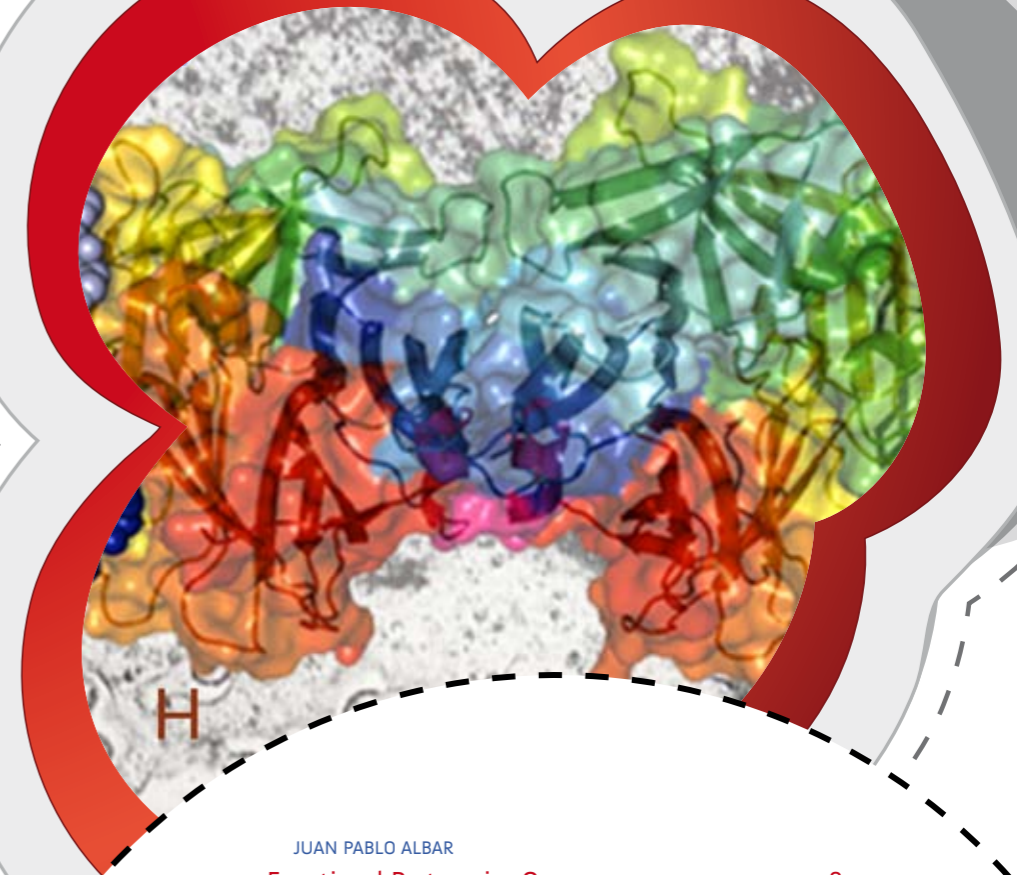
NINJA recruits TOPLESS to various pathways



Macromolecular Structures

The Department of Structure of Macromolecules uses a variety of experimental and computational approaches to study structural and functional properties of biological macromolecules and macromolecular assemblies at different levels of complexity.

Several groups are working on the analysis of cell-cell interactions (with emphasis on receptors of the immune system), and on the virus-cell interplay at distinct stages, including entry, replication, assembly and maturation. These groups work with various approaches, with special emphasis on X-ray crystallography, advanced electron and X-ray microscopy, and tomography. Cell organelles such as the centrosome, centrosomal complexes, chaperones and their cofactors, diverse nanomachines and virus-related complexes are under detailed analysis using correlative approaches, including X-ray crystallography and advanced cryo-electron 3D-microscopy methods. These studies involve extensive development of image acquisition and processing tools to achieve improved resolution. The department has incorporated nanoscopic approaches (optical and magnetic tweezers) to study properties of molecular motors involved in DNA repair and replication as well as other important macromolecular assemblies. The interplay between experimental and computational approaches is an important activity in the department. We also perform research on functional and clinical proteomics, functional characterisation of genes, proteins and protein expression in different experimental conditions and at increasing complexity levels.



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128

JOSE MARÍA CARAZO
Biocomputing Unit

130

JOSÉ LÓPEZ CARRASCOSA
Structure of Macromolecular Complexes

132

JOSÉ M. CASASNOVAS
Cell-Cell and Virus-Cell Interactions

134

JOSÉ RUIZ CASTÓN
Structural Biology of Viral Macromolecular Assemblies

136

JOSÉ JESÚS FERNÁNDEZ
Computational Methods for 3D Electron Microscopy

138

FERNANDO MORENO HERRERO
Molecular Biophysics of DNA Repair Nanomachines

140

ALBERTO PASCUAL-MONTANO
Functional Bioinformatics

142

CRISTINA RISCO ORTIZ
Cell Structure Lab

144

CARMEN SAN MARTÍN PASTRANA
Structural and Physical Determinants of Viral Assembly

146

MARK J. VAN RAAIJ
Structural Biology of Viral Fibres

148

JOSÉ M^a VALPUESTA & JAIME MARTÍN-BENITO
Structure and Function of Molecular Chaperones

150

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Proteomics is a technology-driven research area in which mass spectrometry and protein separation techniques are evolving in conjunction with powerful computational and bioinformatics tools

Advances in the standardisation of proteomic workflows and data formats are nonetheless developed at a suboptimal level. In recent years, the CNB functional proteomics group has targeted differential protein expression in a variety of tissues, cell types and organisms after various experimental treatments/conditions, and subsequent protein identification using mass spectrometry.

We have faced these proteomics challenges as follows:

- 1) The CAM project "Towards Functional Proteomics: an approach integrating Proteomics, Bioinformatics and Structural Biology" (CAM P2006-GEN-0166 2007-10) allowed us to partially characterise subcellular interactions between centrosomal proteins, as well as to assess macromolecular complex components by interactomics techniques based on affinity tags, stable isotopic labelling, mass spectrometry and peptide array approaches. These are considered key issues of the so-called functional proteomics.
- 2) Computational proteomics encompasses analysis of data from large-scale experiments and meta-annotation of proteins and protein complexes, including prediction of protein function, localisation and molecular features. The functional proteomics group has been involved in 1) application of probability-based methods for large-scale peptide and protein identification from tandem mass spectrometry data, 2) implementing methods for data mining visualization (PIKE tool, available at <http://proteo.cnb.csic.es/pike>), and 3) proteomics standard data formatting, reporting, storage and exchange (MIAPE generator web tool, available at <http://www.proteored.org>).

org/MIAPE). The European project "International Data Exchange and Data Representation Standards for Proteomics" (ProteomeXchange) EU FP7-HEALTH-2010 and ProteoRed support most computational proteomics activities of our group.

- 3) Quality control, standardisation, reproducibility and robustness of proteomics workflows are issues that have been evaluated through the participation in multi-laboratory studies within the ProteoRed project (GE 2005X747_3) led by our group.
- 4) Clinical proteomics, including the development of protein profiling methods and biomarker discovery tools for diagnostic and prognostic purposes, is a new and rapidly evolving field. Our group leads the ISCIII project "Novel Diagnostic and Prognostic Proteins Search in Cardiovascular Diseases by Proteomic Analysis" (PI071049), in which we have applied differential quantitative proteomics using label-free and chemical labelling, mass spectrometry and bioinformatics tools to find potential plasma biomarkers associated to acute myocardial infarction. Several candidates are being validated by targeted proteomics and other techniques.

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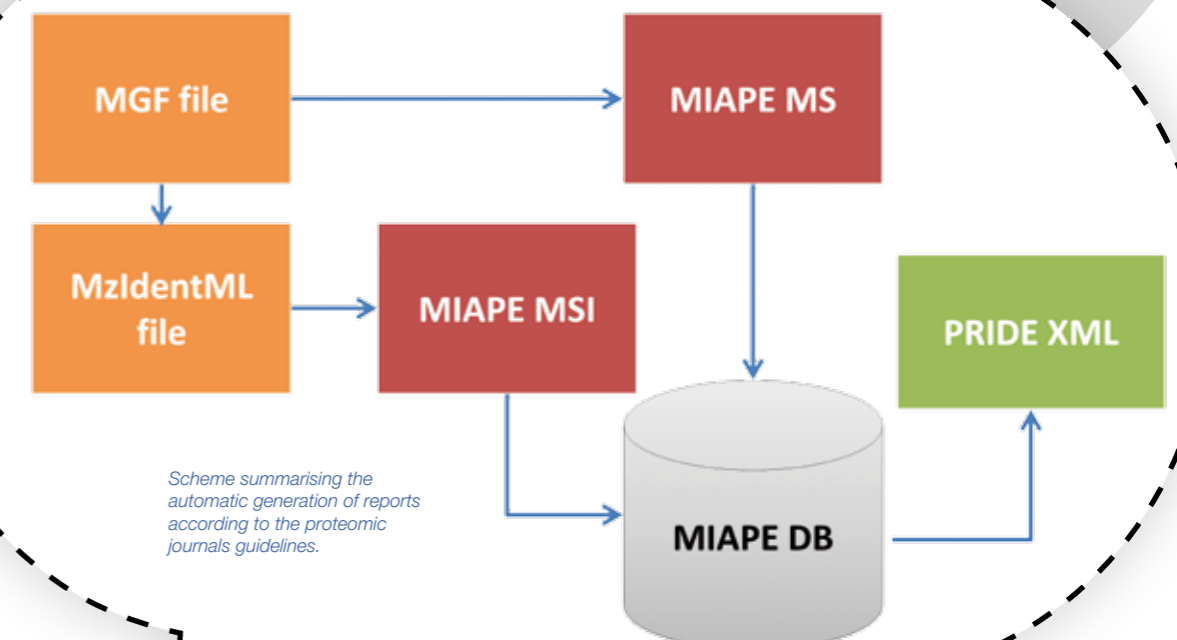
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Scheme summarising the automatic generation of reports according to the proteomic journals guidelines.

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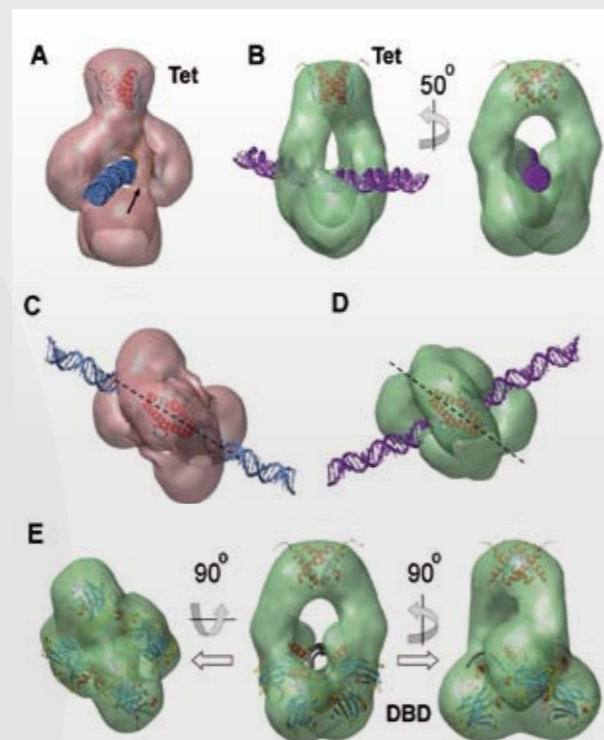
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Our group focuses in the area of three-dimensional (3D) electron and X-ray microscopy, specifically developing new image processing approaches and applying them to challenging experimental biological systems. Consequently, we are a quite interdisciplinary group, where engineers, mathematicians, physicists, biologists and chemists find a common place to work.



The way electron and x-ray microscopes delivers 3D information is by a “tomographic” process by which thousands of different 2D images are combined into a 3D volume. The mathematics foundation of this combination are well routed in the area of inverse problems and are essentially the same when applied to human beings in a medical CT scanner than when applied to a macromolecular complex more than a hundred million times smaller. However, the world at the scale of the nanometer presents unique challenges of its own. Chief among them is the extraordinary flexibility of the molecular machines in charge of performing key tasks

inside the cell. Indeed, their detailed composition and shape usually changes while performing their function. Obviously, we are not interested in obtaining a “blurred” 3D structure, average of the many shapes a given “nano machine” may have, but a detailed 3D structure of each of these stages. In this way we enter the world of 3D “classification” and “reconstruction”, starting from thousands of very noisy images.

In order to address this problem we have developed new mathematical approaches that are able to distinguish subtle change variations at the nanometer scale, classifying the microscopic images and rendering new biological insights. Then, we port these development into our widely distributed image processing suite, named XMIPP, making them accessible as part of our committeemen in the context of the European project for strategic research infrastructures in structural biology, named Instruct.

Obviously, we are eager to test these developments into challenges biological systems, either systems we work internally at the laboratory, or working with external collaborators. In both cases the aim is to deliver new biological information through this new capability to handle conformational changes in 3D, proving “snap shot” of the actual “work” at the nano scale.

Recently we have entered the field of “Soft X-ray Tomography”, expanding the approaches just described to the “meso scale”, visualizing complete cells using synchrotron radiation. Indeed, Spain now has the third microscope of its class at the Spanish synchrotron ALBA, opening new perspective in biology we are actively getting involved with.

SELECTED PUBLICATIONS

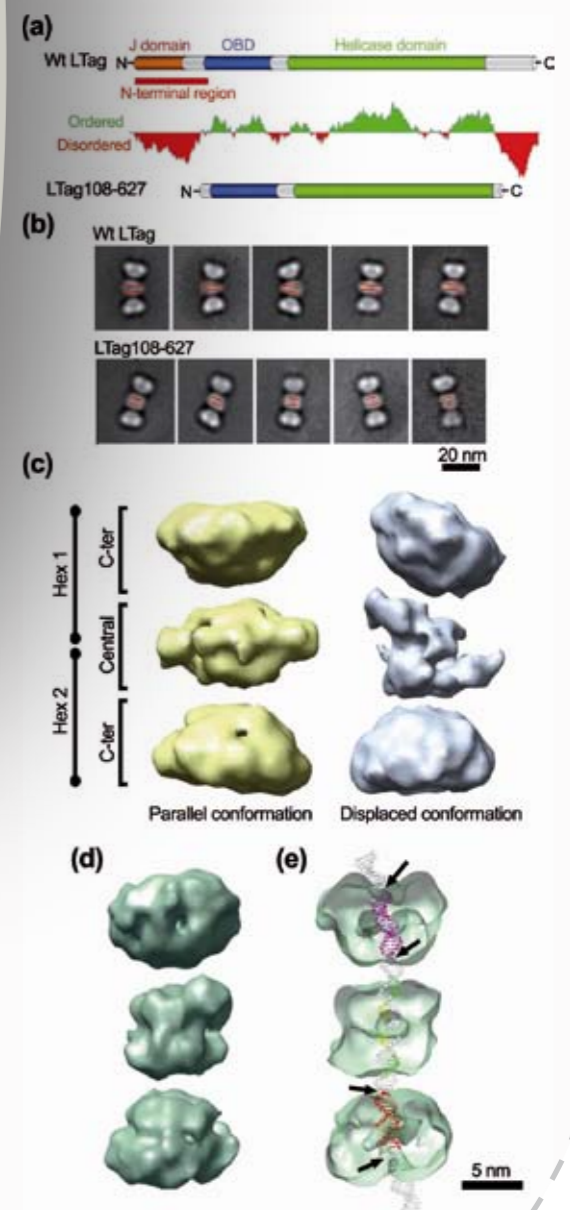
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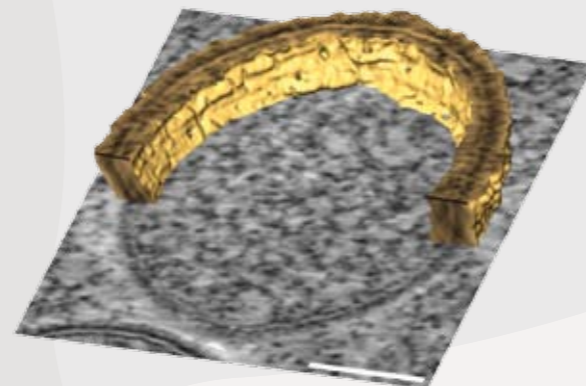
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Structure of Macromolecular Complexes

The group is working on the study of virus assembly and maturation using an integrative approach of microscopic, biochemical and biophysical methods.

Our aim is to determine the molecular bases of the interaction of macromolecules and macromolecular complexes to yield functional biological machineries. We have centred our interest on the way viral proteins assemble into intermediate structures that are further matured to yield fully infective viral particles. Our model systems are bacteriophages (T7 and $\Phi 29$), and eukaryotic viruses such as vaccinia. Using bacteriophages, we have reached subnanometer resolution by electron cryo-microscopy and three-dimensional reconstruction. The combination of these structures with computational modelling has led to the definition of the molecular basis of capsid expansion and stabilization characteristic of certain virus families.



Tomographic reconstruction of a maturation intermediate of vaccinia within the infected cell. A plane of the tomographic reconstruction (gray levels) is coupled to a section of the tomographic reconstruction of the viral envelope.

The intracellular maturation of eukaryotic viruses is currently studied using vaccinia and other complex viruses. We are following the rearrangements involved in the sequential generation of intermediate morphogenetic

particles within the cytoplasm by combining electron cryo-microscopy and low temperature processing methods to retrieve three-dimensional tomographic reconstructions of virus-infected cells in a native preservation state. The tomographic volumes are used as templates to map by computer modelling other structural data, from x-rays or single particle cryo-microscopy, to yield high resolution topographic maps. As electron microscopes have limited penetration power, extension of the use of tomographic methods to the cell environment demands other types of microscopy. We are developing x-ray microscopy methods to obtain three-dimensional cryo-tomographic reconstructions of whole cells at intermediate resolutions between electron and light microscopy. These developments are coordinated with the set-up of the microscope beam line at the Spanish synchrotron ALBA.

We are also currently exploring the potential of combining different biophysical methods for the analysis of biological material at the nanoscopic level. We have worked intensively on the use of atomic force microscopy to study the nanomechanical properties of viral capsids, and the structure of other protein-nucleic acids complexes. Together with the group of Jose M. Valpuesta (CNB), we are developing optical and magnetic tweezers to study molecular motors at the single molecule level, as well as to explore their potential for cell organelle manipulation.

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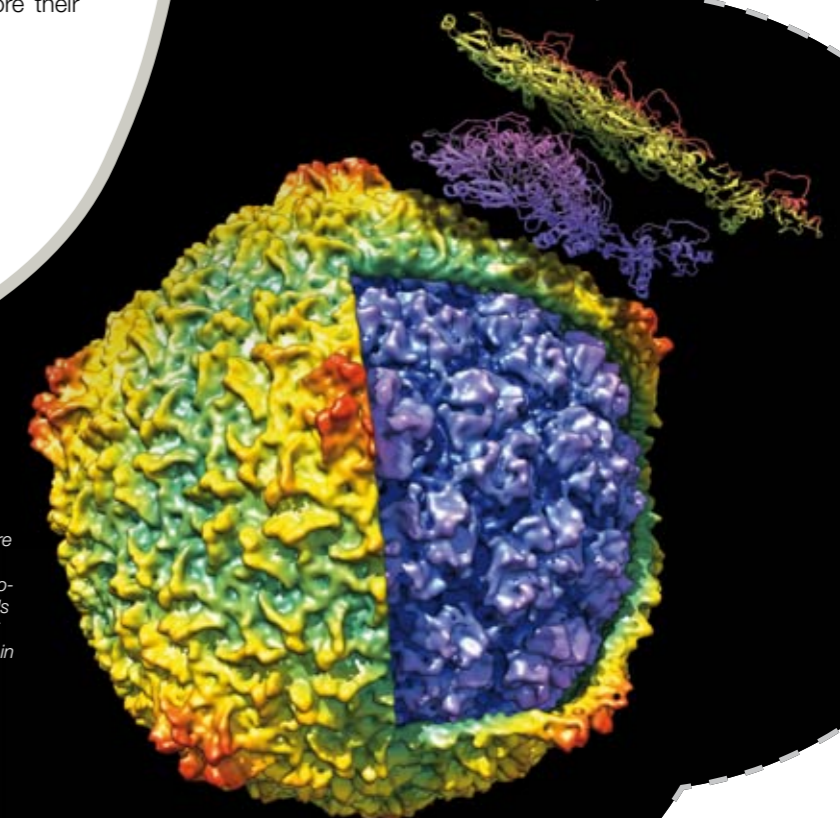
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Three-dimensional reconstruction of bacteriophage T7 mature capsid (yellow) and prohead (blue) obtained by electron cryo-microscopy. The atomic models correspond to the threading of the capsid major protein gp10 in the mature capsid (yellow) and the prohead (blue).



Cell-Cell and Virus-Cell Interactions

Macromolecular Structures

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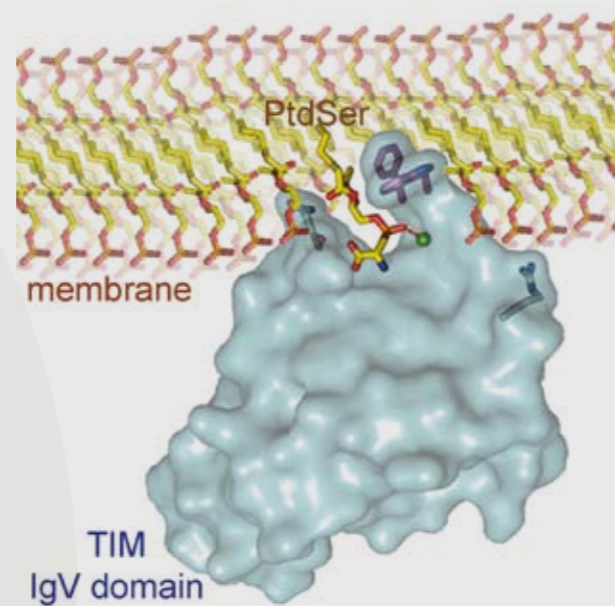
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A large variety of glycosylated proteins that participate in cell-cell and virus-cell interactions populate the cell and viral membranes. The surface proteins allow the cell to communicate with its environment and participate in cell migration and in virus entry processes.



Our group analyses receptor-ligand recognition using biochemical techniques and X-ray crystallography, studying receptors of the immune system, some of which have been subverted by viruses to enter host cells. Our research also focuses on receptor-mediated virus entry into cells, with viruses of medical interest that use the receptor molecules for attachment and cell membrane penetration. We study receptor recognition and the dynamics of virus entry with rhinovirus, measles virus and coronavirus. Our research provides new knowledge on cell adhesion and virus entry processes, and it can lead to the development of anti-viral therapies and treatments for immune disorders. Below we highlight some of our recent results, related to both immune processes and viral infections.

TIM proteins: a family of PtdSer receptors that regulate immunity

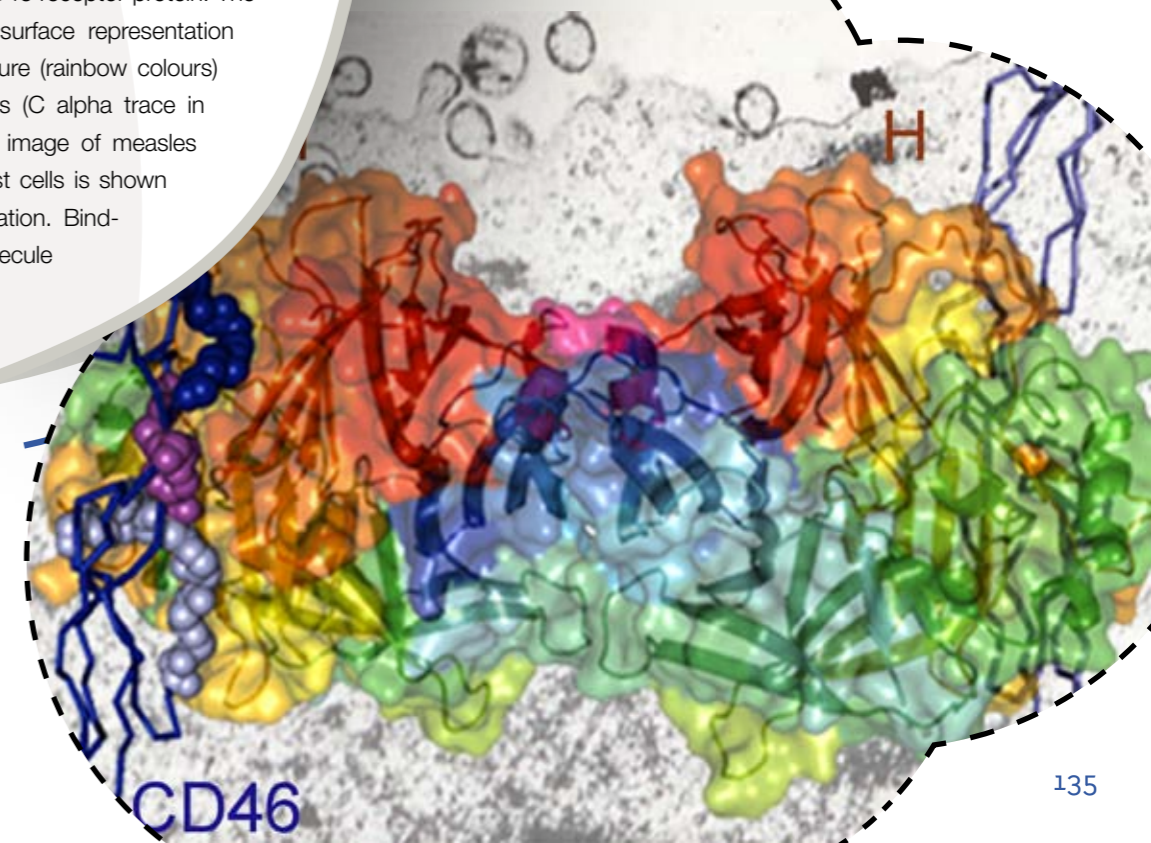
The T cell/transmembrane, immunoglobulin and mucin domain (TIM) gene family plays a critical role in regulating immune responses, including transplant tolerance, autoimmunity, the regulation of allergy and asthma, and the response to viral infections. We demonstrated that the TIM proteins are pattern recognition receptors, specialized in recognition of the phosphatidylserine (PtdSer) cell death signal. The figure on the left shows a surface representation of the structure of the mTIM-4 IgV domain bound to PtdSer in a lipid membrane bilayer, which was determined in our laboratory. Cells expressing TIM-1, TIM-3 and TIM-4 can mediate elimination of apoptotic cells, which display PtdSer on their outer membrane leaflet. This biological process is essential for maintaining tissue homeostasis and prevention of autoimmunity and inflammatory reactions.

Measles virus binding to the CD46 cell receptor

Measles virus (MV) has a glycoprotein, known as haemagglutinin (H), anchored to its lipid envelope. The H protein is specialized in the recognition of cell surface receptor molecules such as CD46; this process is essential for virus entry into host cells. We have been able to visualize this molecular event by determining the crystal structure of the measles virus H protein bound to the CD46 receptor protein. The image on the right shows a surface representation of the dimeric H protein structure (rainbow colours) bound to two CD46 molecules (C alpha trace in blue). An electron microscopy image of measles virus particles entering into host cells is shown below the structural representation. Binding to the CD46 receptor molecule is essential for MV entry into human cells and it begins the virus infective cycle.

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Macromolecular Structures

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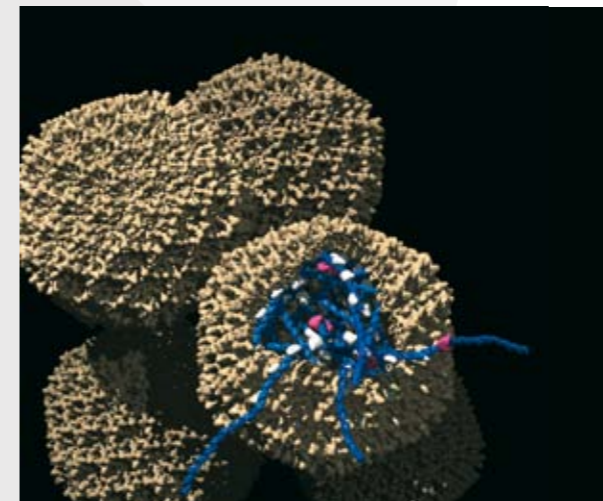
Structural Biology of Viral Macromolecular Assemblies

Our studies attempt to elucidate structure-function-assembly relationships of viral macromolecular assemblies by three-dimensional cryo-EM in combination with X-ray structures.

We are investigating several viral systems with different levels of complexity: double-stranded (ds)RNA viruses such as infectious bursal disease virus (IBDV) and *Penicillium chrysogenum* virus, and virus-like particles of a single-stranded RNA virus, rabbit hemorrhagic disease virus. Understanding the structural basis of the polymorphism of the coat proteins has been our major goal. The general rules that govern coat protein conformational flexibility can be extended to other macromolecular assemblies that control fundamental processes in biology. We also focus on the structural basis of dsRNA virus replication. All dsRNA viruses, from the mammalian reoviruses to the bacteriophage phi6, share a specialised capsid involved in transcription and replication of the dsRNA genome.

The IBDV has a T=13I capsid built of a single protein, VP2 (441 residues). VP2 is initially synthesised as a 512-residue precursor, pVP2. The conformational switch responsible for the VP2 structural polymorphism resides in the segment 443-453, which forms an amphipathic helix. The other major protein, VP3, participates in capsid assembly as a scaffolding protein. This process is mediated by electrostatic interactions of the VP3 acidic C-terminal region with the basic side of the amphipathic helix. In addition, VP2 has Asp431-mediated endopeptidase activity that does not disrupt the Ala441-Phe442 bond. Our structural analysis also showed an unusual feature for IBDV: the cargo space of the capsid is much larger than is necessary to enclose a single genome copy. Indeed, IBDV is an icosahedral polyploidy dsRNA virus that can package more than one complete genome copy. Multiploid IBDV particles propagate with higher efficiency than haploid virions. VP3 is also an RNA-binding protein and is closely associated with the viral genome, forming ribonucleoprotein (RNP) complexes. VP3 renders these RNP less accessible to nucleases. RNP complexes are functionally competent for RNA synthesis in a capsid-independent manner.

We analysed the capsid structure of a fungal dsRNA virus, *Penicillium chrysogenum* virus. This capsid shows that the asymmetric unit is formed by a repeated helical core, indicative of gene duplication. This basic repeated motif could provide insight into an ancestral fold and show structural evolutionary relationships of the dsRNA virus lineage. Our results indicate that a dimer as the asymmetric unit is a conserved arrangement favourable for managing replication and genome organization of dsRNA viruses.



Infectious bursal disease virus (IBDV), an avian pathogen, is a non-enveloped icosahedral virus that has a bisegmented dsRNA genome (blue) enclosed within a single-layered capsid with T=13I geometry (orange). dsRNA is bound to a nucleocapsid protein (VP3, white) and the RNA polymerase (VP1, pink). IBDV is the first icosahedral virus to be described that can package more than one complete genome copy.

SELECTED PUBLICATIONS

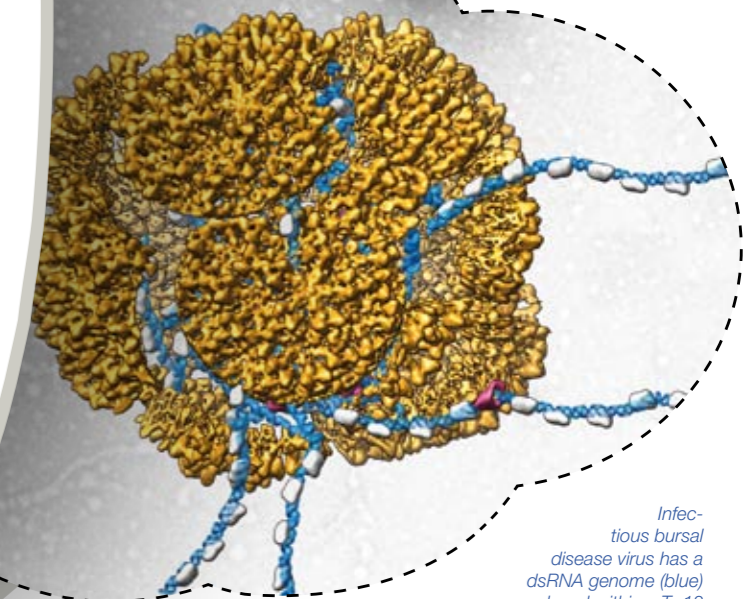
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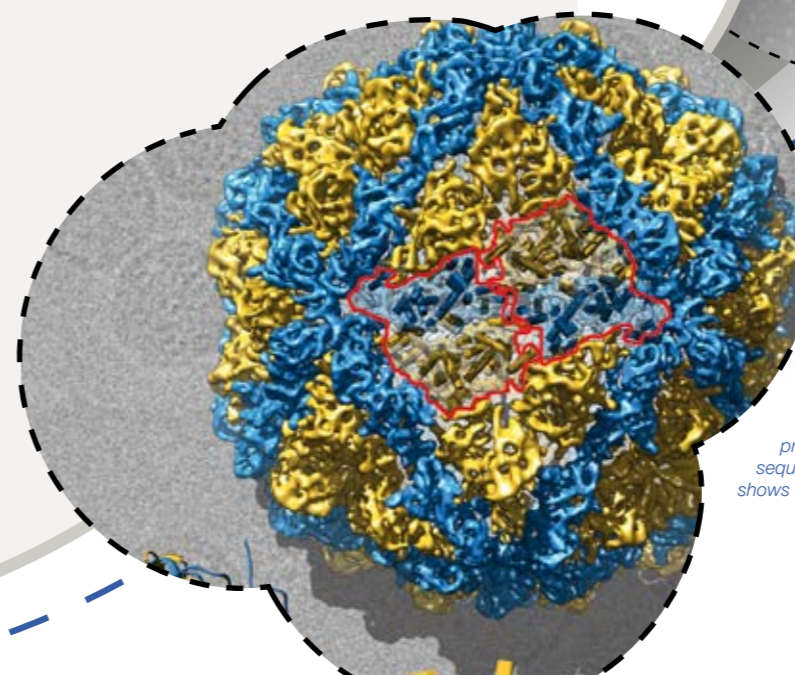
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Infectious bursal disease virus has a dsRNA genome (blue) enclosed within a T=13 capsid (yellow). The genome, organised as ribonucleoprotein complexes bound to a nucleocapsid protein (white) and the RNA polymerase (pink), can be released by mild disruption of viral particles (background) and is functionally competent for RNA synthesis.



Structure of *Penicillium chrysogenum* virus (PcV), a fungal double-stranded RNA virus. Three-dimensional cryo-EM reconstruction of the PcV virion at 8 Å resolution, which has a T=1 capsid formed by 60 copies of a single polypeptide. Boundaries for two-capsid proteins are outlined in red and their secondary structural elements are highlighted; each subunit has two similar domains (yellow and blue), suggesting ancestral gene duplication. This basic repeated motif could provide insight into an ancestral fold and joined motifs, with the same fold but lacking sequence similarity, have been described in other capsid proteins. The background shows PcV particles frozen in vitreous ice.

Macromolecular Structures

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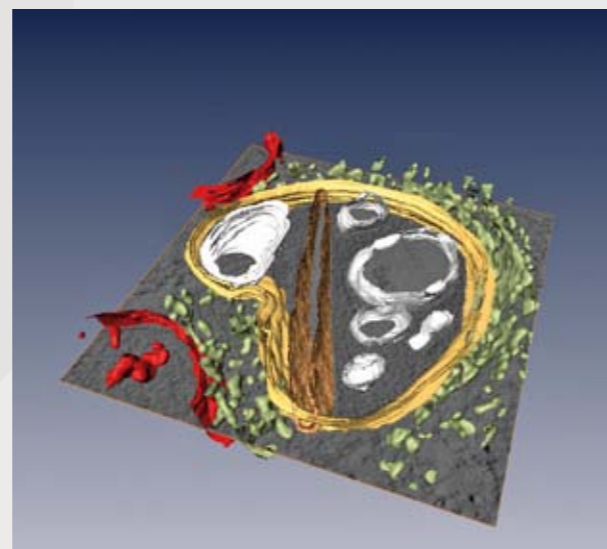


Computational Methods for 3D Electron Microscopy

Knowledge of the structure of biological specimens is essential to understanding their functions at all scales.

Electron microscopy (EM) combined with image processing allows the investigation of the three-dimensional (3D) structure of biological specimens over a wide range of sizes, from cell structures to single macromolecules, providing information at different levels of resolution. Depending on the specimen under study and the structural information sought, different 3D EM approaches are used. Single particle EM makes it possible to visualize macromolecular assemblies at subnanometer or up to near-atomic resolution. Electron tomography turns out to be a unique tool for deciphering the molecular architecture of the cell. In all cases, the computational methods of image processing play a major role. Computational advances have contributed significantly to the current relevance of 3D EM within structural biology.

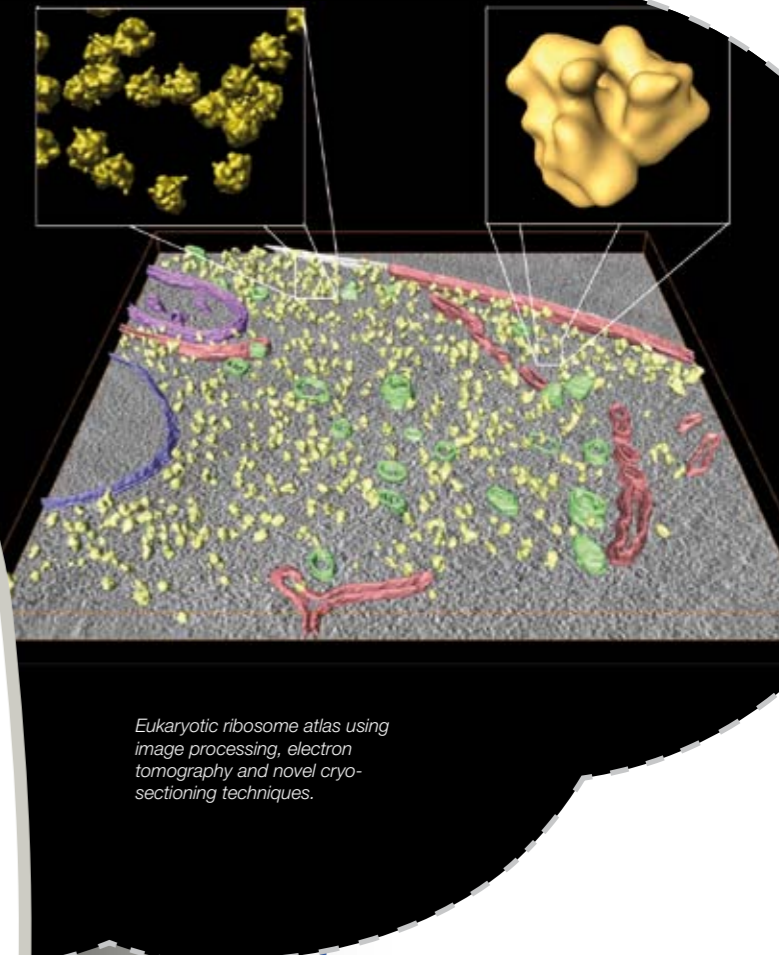
Rubella virus factory visualized in three dimensions using electron tomography.



Our research interests are focused mainly on the development of image processing methods for structural analysis of biological specimens by 3D EM. We also devise high performance computing strategies to approach some of the computational challenges in this field. The next step is the application of these computational developments to the study of biological problems of interest. We are interested in the application of electron tomography to explore alterations in subcellular architecture under normal and pathological conditions, particularly in neurodegenerative diseases. In addition, we collaborate with other national and international groups in experimental structural studies.

In the last few years, we have developed new image processing methods for electron tomography, specifically for three-dimensional reconstruction and noise filtering. As far as structural studies are concerned, we have made significant advances towards the construction of an atlas of the eukaryotic ribosome using image processing, electron tomography and novel cryosectioning techniques, in collaboration with the Carrascosa and Peters groups. We have also collaborated with the Risco group in the structural characterization of rubella virus factories by electron tomography.

Dr. M. R. Fernández Fernández recently joined the group. She worked previously on different aspects of the molecular and cellular biology of Huntington's disease neurodegeneration. We are now conducting a project focused on the use of electron tomography to understand structural alterations at the subcellular level in Huntington's disease.



Eukaryotic ribosome atlas using image processing, electron tomography and novel cryosectioning techniques.

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Macromolecular Structures

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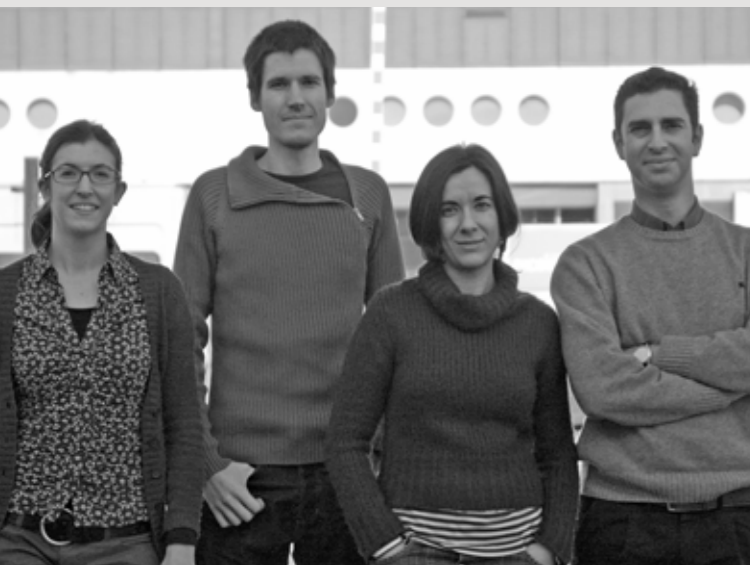
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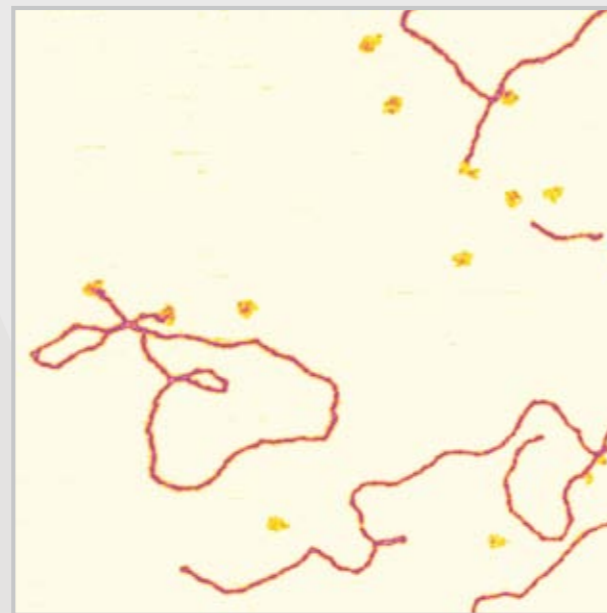
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Molecular Biophysics of DNA Repair Nanomachines

The molecular biophysics group joined the CNB in 2009 to develop single-molecule techniques and to study the mechanisms of molecular motors and DNA repair protein machines.



AFM image showing AddAB proteins (yellow) and DNA molecules (red). Often AddAB appears bound at the end of a DNA molecule. Image size, 1 micrometer.

DNA breaks are a potentially catastrophic form of DNA damage that can lead to cell death, premature ageing or cancer. Consequently, nature has developed robust mechanisms to repair DNA breaks that involve dynamic, large-scale DNA manipulations by molecular motors and protein machines. Understanding how these machines work in model organisms will underpin a deeper knowledge of the basic enzymology of DNA repair, and provide important insights into the molecular basis of carcinogenesis.

Over the last two years, a large effort has been made to set up the techniques and to establish research lines that are now starting to give results. During this short time, we have built a magnetic tweezers (MT) instrument that can manipulate single DNA molecules and measure force and torque applied by molecular motors. The group will also have a custom-adapted atomic force microscope (AFM) and an optical tweezers (OT), whose construction is expected to be finished in 2011. We follow two main research lines, supported by a solid collaboration with the group of Dr. M.S. Dillingham at Univ. Bristol, i) to study the dynamics and mechanisms of the helicase-nuclease AddAB from *Bacillus subtilis* and ii) to study the mechanisms of cohesion and condensation of DNA by structural maintenance of chromosome proteins. Using AFM and MT, we have been able to monitor in real time the translocation of AddAB—a molecular motor involved in DNA-end processing in homologous recombination— and to visualise individual proteins in the act of moving along a single DNA molecule. Surprisingly, we found that the coupling between translocation and unwinding in a helicase is a complex parameter and that unwinding is promoted by intermediates that differ from the classical “Y” shape. Moreover, the helicase activity of AddAB was activated by encounter with a specific sequence in the DNA track and that generated a loop structure that we captured using our single-molecule techniques.

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Yeeles J, Longman E, Moreno-Herrero F, Dillingham MS (2010) Activation of a Helicase Motor Upon Encounter With a Specific Sequence in the DNA Track. *Biophys J* 98:66a.

AFM image showing AddAB caught in the act of translocating along DNA. Unwound strands immediately re-anneal behind the translocating motor. Dark structures are single-stranded DNA with bound AddAB proteins. Image size, 1 micrometer.

Functional Bioinformatics

Macromolecular Structures

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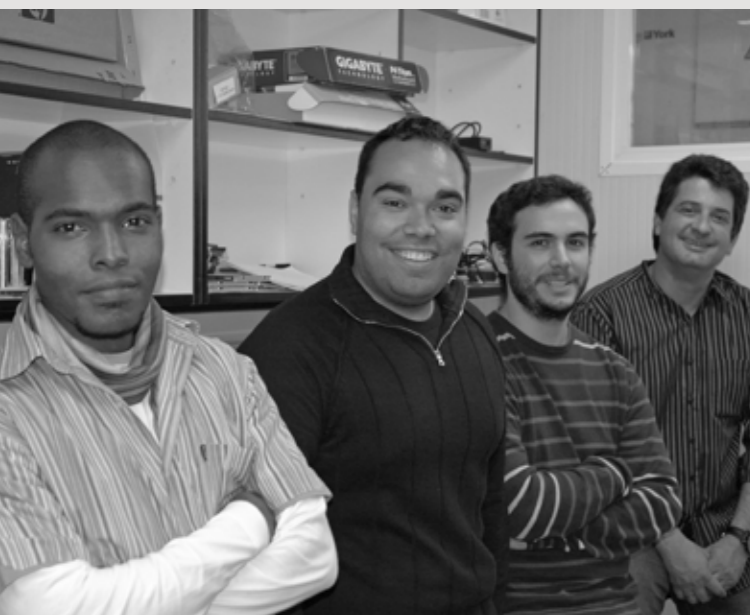
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To help in understanding the biology that underlies experimental settings, our group is dedicated to the development of new methods and analytic techniques to solve specific biological questions.

We concentrate on functional bioinformatics, whose focus is on the functional characterization of genes and proteins in different experimental conditions. We also develop new methods for the analysis and interpretation of biological data, and centre on three major areas: analysis of gene expression information, functional analysis of annotations and scientific literature analysis.

For gene expression analysis, we have developed several techniques to bicluster data (find functional patterns of small set of genes that show coherent expression in a small subset of experimental conditions). This development is based a novel matrix factorization approach that can produce not only a clustering the genes and experimental conditions simultaneously, but also aid in its interpretation.

In this line, we have also developed several new techniques for the functional characterization of lists of genes or proteins. The novelty of our proposal lies in the combination of several information sources and determination of which combination of functional annotations is significantly enriched in the gene or protein list. This has opened a new research area known as modular functional enrichment.

These studies are complemented by one of the richest sources of biological information currently available: the scientific literature. We have developed several methods and tools with which to analyse the set of Medline abstracts related to lists of genes and proteins, and to summarize

its content into semantic features that can later be integrated into functional analysis. In this way, a global understanding of biological events is possible.

A large set of high-quality bioinformatics software has also been developed, published and made available to the scientific community. The figure summarises the developments of our group in functional bioinformatics. More details can be found at <http://bioinfo.cnb.csic.es>

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<http://bionmf.cnb.csic.es>

bioNMF is a web-based tool for performing non-negative matrix factorization on biological data, including clustering, biclustering and sample classification.



<http://genecodis.cnb.csic.es>

GeneCodis is a tool for the functional analysis of large lists of genes. It determines combinations of annotations that are significantly associated to a list of genes.



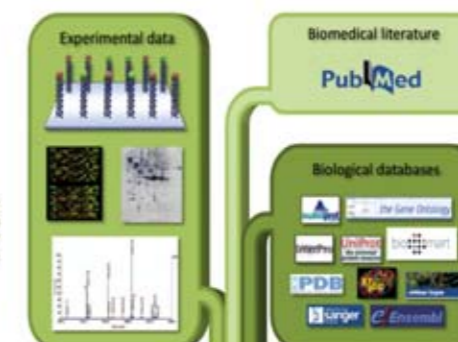
<http://sent.cnb.csic.es>

SENT explores the biomedical literature associated to a gene list and summarize its contents as semantic features. It also clusters genes according to their semantic content.



<http://moara.cnb.csic.es>

Moara is a Java Library for entity recognition or mention (detection of genes and proteins names) and normalization (linking detected genes or proteins with their corresponding identifiers) in free-form scientific documents.



<http://centrosome.cnb.csic.es>

Centrosome.db is a database that contains a set of human genes encoding proteins that are localized in the centrosome. It offers different perspectives to study the human centrosome, including evolution, function, and structure.



<http://engine.cnb.csic.es>

Engine is a platform-independent exploratory data analysis tool for gene expression data. It integrates a variety of analysis tools for visualizing, pre-processing and clustering expression data.



<http://marq.cnb.csic.es>

MARQ (Microarray Rank Query), search and query GEO database to find similar or opposite gene expression signatures to those than a query dataset. It also contains meta-analysis functionality.



<http://chipcodis.cnb.csic.es>

CHIPCodis is a web-based tool for mining complex regulatory systems in yeast by concurrent enrichment analysis of chip-on-chip data.



<http://proteopathogen.cnb.csic.es>

Proteopathogen is a database intended to compile proteomics experimental data and to facilitate storage and access to a range of data. It is currently focused on *Candida albicans* and its interaction with macrophages

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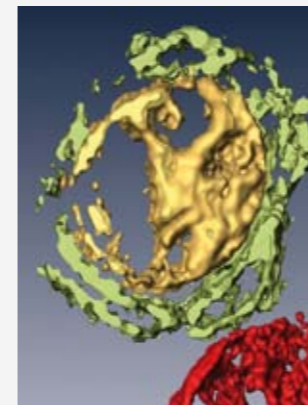
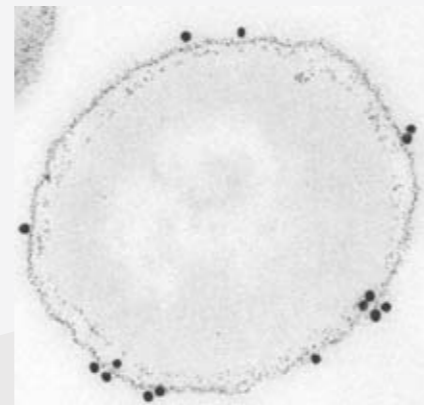
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RUBV factory in 3D as studied by electron tomography (right) and detection of Hfq protein in *E. coli* with the MT clonable tag (left). Hfq molecules (small particles) are detected in the inner membrane, outer membrane (immunogold-labelled, large particles) and the periplasm between them.

Viral factories are complex structures built by many different viruses as a physical scaffold for viral replication and morphogenesis.

Signalling events involved in the assembly of viral factories are mainly unknown. We are interested in characterizing the factories of several RNA viruses that are important pathogens for humans, simultaneously using viruses as very valuable tools to study cell architecture. One of our main goals is to develop new methods for specific detection of macromolecules in 3D maps of whole cells. This will permit progress in one of today's most ambitious challenges in structural biology: elaboration of molecular atlases of cells to understand the structure-function relationships that underlie cell functions. Within this context, our activities in 2009 and 2010 are summarized as follows:

- Correlative microscopy methods helped us to understand the dynamics of arbovirus infection in mosquito cells. We detected new anti-viral mechanisms that maintain infection under control in these cells (López-Montero and Risco, Cell Microbiol 2010).
- 3D maps of factories built by rubella virus have been obtained by electron tomography and reveal how cell organelles are modified and interact with each other in the factory scaffold (Fontana et al., Virology 2010). This work was done in collaboration with Dr. José J. Fernández in the Department of Structure of Macromolecules (CNB).

- After validating the first clonable tag for electron microscopy that works in live cells, the "GFP" of ultrastructural analysis, we applied this new methodology to the study of proteins in bacteria (Diestra et al., J. Struct. Biol. 2009; Diestra et al., PLoS ONE 2009) and eukaryotic cells (Risco and Sanmartín, patent, 2010). This new technology based on the metal-binding protein metallothionein (MT) could be an important step that will provide us with a completely new vision of structure-function relationships in complex biological systems.

We are currently studying 1) the nature of contacts between cell organelles in viral factories, 2) how membrane-associated arrays of viral polymerases work and release the replicated viral genomes, 3) how replication and assembly are spatially connected inside viral factories, 4) the major structural changes triggered in immature viral particles to become infectious virions inside the factories, and 5) applications of new synthetic tags for correlative microscopy and molecular mapping in 3D EM.

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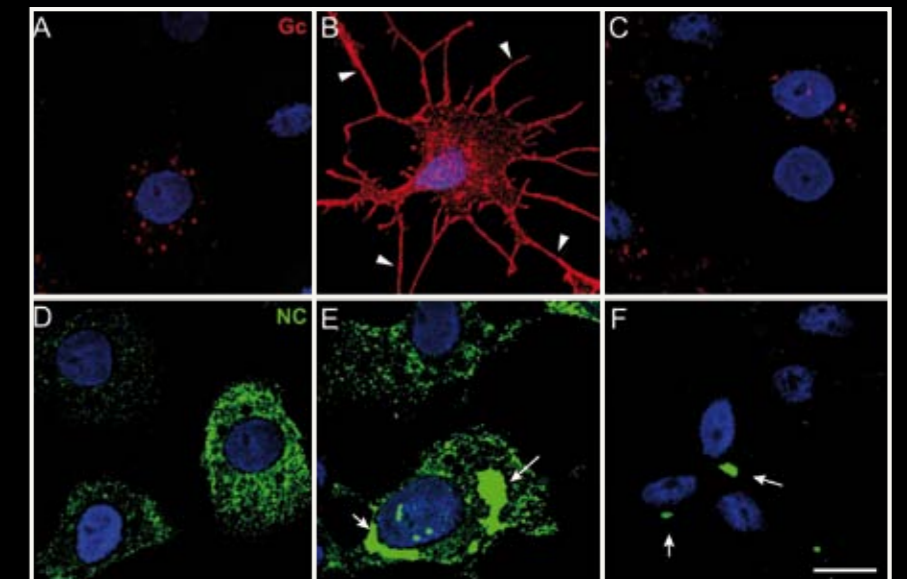
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López-Montero N, Risco C (2011) Self-protection and survival of arbovirus-infected mosquito cells. **Cell Microbiol** 13:300-15.

PATENT

Cristina Risco and Eva Sanmartín. Marcador clonable para su uso en microscopía. Patent application: **P201031880** (Dec 2010). CSIC

Changes in the distribution of Gc (red) and NC (green) viral proteins in Bunyavirus-infected C6/36 mosquito cells. NC protein is progressively trapped in N-bodies (white arrows in F).



Macromolecular Structures

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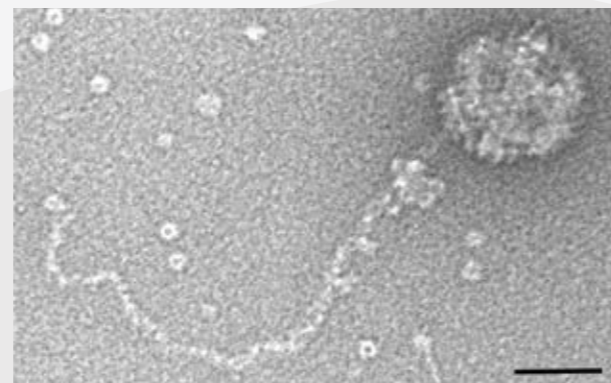
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Inna Romanova

Structural and Physical Determinants of Viral Assembly

We are interested in the structural and physical principles that govern assembly and stabilization of complex viruses.

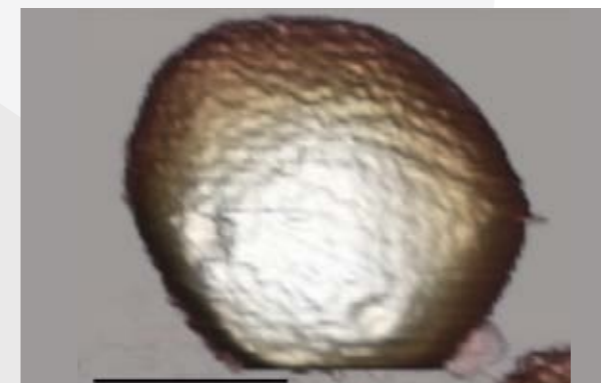


As a model system we use adenovirus, a challenging specimen of interest both in basic virology and nanobiomedicine. We approach the problem from an interdisciplinary point of view, combining biophysics, computational, structural and molecular biology techniques. Sharing expertise and resources is a pillar in our work; accordingly, we maintain a variety of intra- and extramural collaborations that enrich the development and interpretation of our work.

Adenoviruses are pathogens of clinical relevance in the increasingly large immunocompromised population. They are also widely used as vectors for gene therapy, vaccination and oncolysis. The viral particle is composed of more than 10 distinct proteins plus the dsDNA viral genome, for a total molecular weight of 150 MDa. During the 2009-2010 period, we determined the structural differences between the mature and immature adenovirus particle. A thermo-sensitive mutant that does not package the viral protease produces viral particles containing unprocessed precursors of several capsid and core proteins. These virions, which represent the immature assembly intermediate, are not infective due to a defect in entry and uncoating. Subnanometer resolution difference maps calculated between mature and immature virus particles, and interpreted with the help of available crystal structures, revealed the conformational changes required to switch the stability requirements from assembly to uncoating during the infectious cycle. In the

Two ways of analysing adenovirus capsid stability. Left, electron microscopy; right, atomic force microscopy (in collaboration with PJ de Pablo, Univ Autónoma de Madrid). Bars represent 50 nm.

immature virus, precursors of minor coat proteins pIIIa, pVIII and pVI increase the network of interactions that hold together the icosahedral protein shell, while core protein precursors pVII and



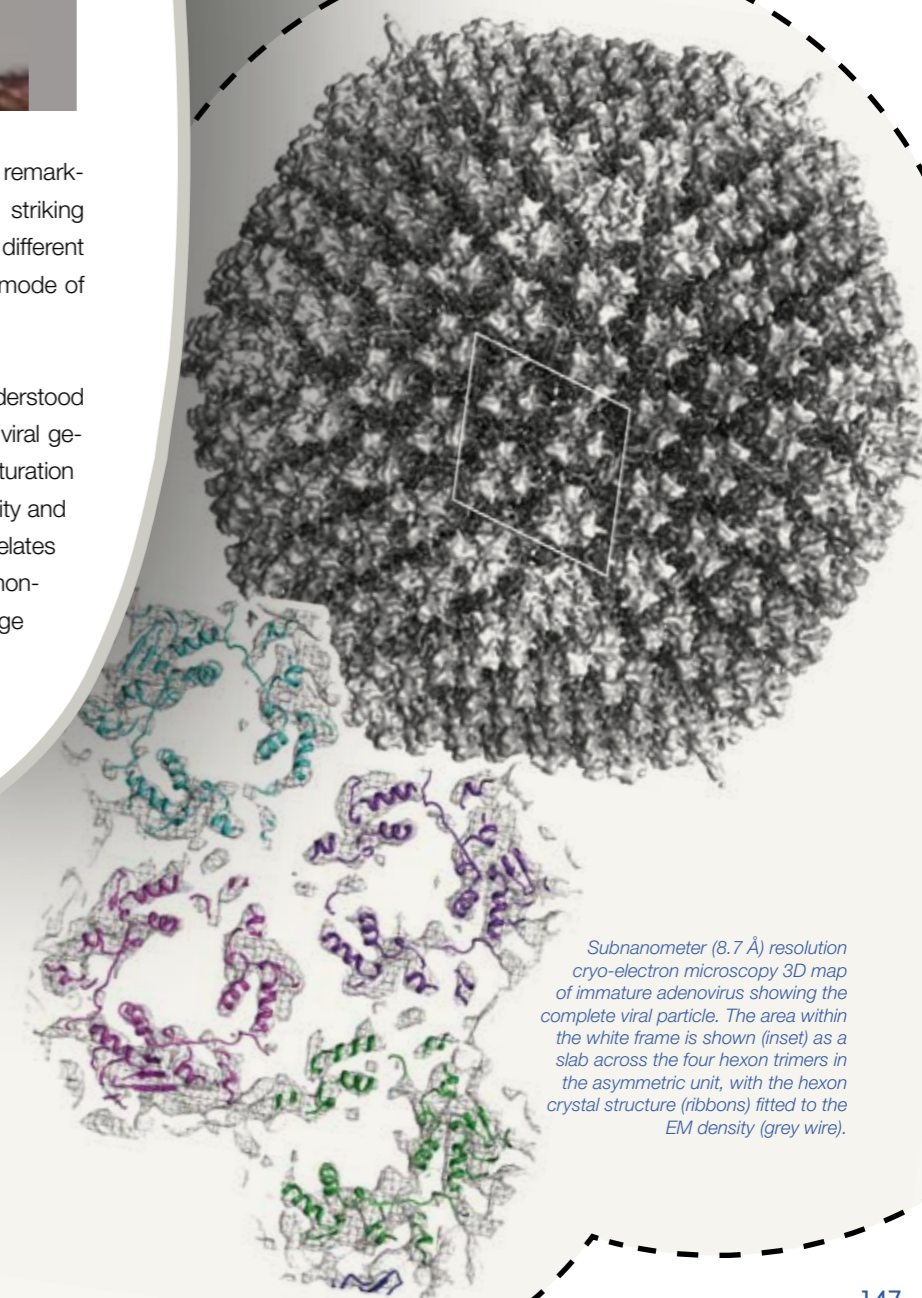
pre-μ act to tightly compact the viral genome into a remarkably stable, spherical structure. Interestingly, this striking change in core organization correlates with a different disassembly pattern, providing a glimpse into the mode of DNA packing within the virion.

Our current research lines focus on the less understood aspects of adenovirus assembly, such as how the viral genome is packaged into the capsid, how virion maturation occurs, the key elements that modulate virion stability and mechanical properties, how adenovirus evolution relates to that of its hosts and finally, the organization of non-icosahedral virion components. Accurate knowledge of adenovirus structure and biology is fundamental to both the discovery of anti-adenovirus drugs and the design of new, efficient adenoviral therapeutic tools.

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Subnanometer (8.7 Å) resolution cryo-electron microscopy 3D map of immature adenovirus showing the complete viral particle. The area within the white frame is shown (inset) as a slab across the four hexon trimers in the asymmetric unit, with the hexon crystal structure (ribbons) fitted to the EM density (grey wire).

Macromolecular Structures

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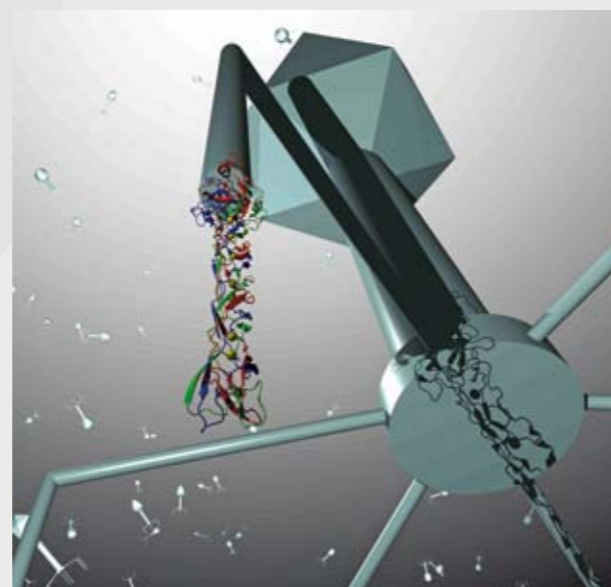


Structural Biology of Viral Fibres

Adenovirus and bacteriophages such as T4, T5, T7 and lambda attach to their host cell via specialised fibre proteins.

These fibres all have the same basic architecture: they are trimeric and contain an N-terminal virus attachment domain, a long thin shaft domain and a more globular C-terminal cell attachment domain. These fibrous proteins are very stable to denaturation by temperature or detergents. In 2010, we determined the structures of the porcine adenovirus type 4 fibre head and galectin domains and of the receptor-binding tip of the bacteriophage T4 long tail fibre protein gp37.

The adenovirus NADC-1 isolate, a strain of porcine adenovirus type 4, has a fibre containing an N-terminal virus attachment region, shaft and head domains and a C-terminal galectin domain, connected to the head by an RGD-containing sequence. The crystal structure of the head domain is similar to previously solved adenovirus fibre head domains, but specific residues for binding coxsackievirus and adenovirus receptor (CAR), CD46 or sialic acid are not conserved. The structure of the galectin domain revealed an interaction interface between its two carbohydrate recognition domains, locating the two sugar binding sites face-to-face. Other tandem-repeat galectins may have the same arrangement. We showed that the galectin domain binds



Schematic image of bacteriophage T4 with the structure of the receptor-binding tip of the long tail fibre highlighted. The phage is viewed from the bottom, i.e. from the side of the bacterium about to be infected (image courtesy of Florencio Pazos of the Sequence Analysis and Structure Prediction Unit, CNB).

carbohydrates containing lactose and *N*-acetyl-lactosamine units. Modification of the galectin domain of this fibre should allow targeting to specific carbohydrate receptors.

Bacteriophages are the most numerous replicating entities in the biosphere, but little high-resolution structural detail is available on their receptor-binding fibres. We solved the structure of the receptor-binding tip of the bacteriophage T4 long tail fibre. It showed an unusual elongated six-stranded anti-parallel β -strand needle domain containing seven iron ions coordinated by histidine residues arranged co-linearly along the core of the biological unit. At the end of the tip, the three chains intertwine to form a broader head domain, which contains the putative receptor interaction site. The structure, a previously unreported beta-structured fibrous fold, provides insights into the remarkable stability of the fibre and suggests a framework through which mutations expand or modulate receptor-binding specificity. Modification of the bacteriophage fibre receptor binding specificities might lead to improved detection and elimination of specific bacteria.

We also collaborated with other research groups and have determined the structures of cyclic antibiotic peptides synthesised by the group of Dr Mark Overhand at Leiden University (The Netherlands) and of bacterial dehydroquinases complexed with inhibitors synthesised by the group of Dr Concepción González Bello of the University of Santiago de Compostela (Spain).

Structure of the galectin region of the porcine adenovirus type 4 fibre illustrating the cooperative nature of tri-*N*-acetyl-lactosamine (yellow) binding by the N- and C-terminal carbohydrate-recognition domains (shown in blue and red, respectively).

SELECTED PUBLICATIONS

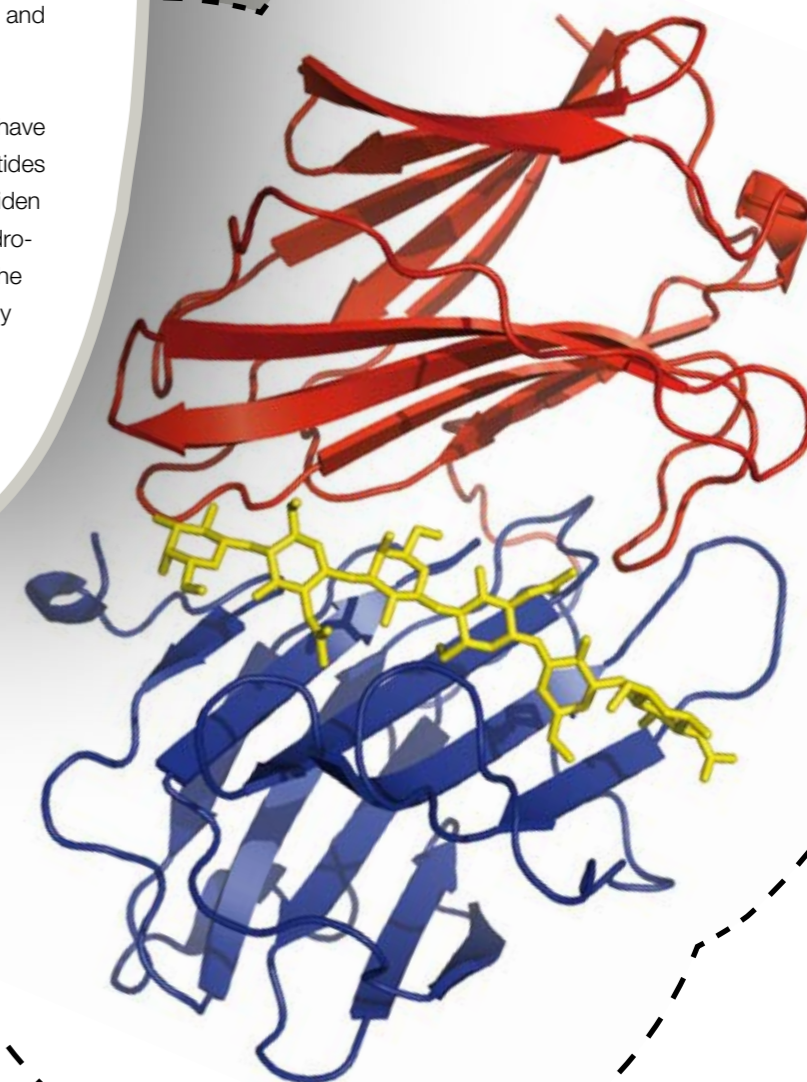
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Macromolecular Structures

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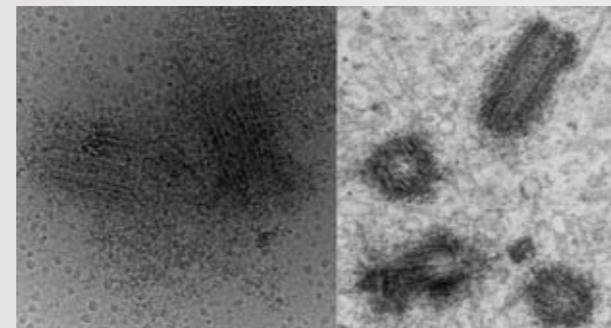
Rocío Arranz
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Structure and Function of Molecular Chaperones

Our group is interested in the structural and functional characterisation of macromolecular complexes, using electron microscopy and image processing techniques as our main tools.

In particular, we are very much interested in the study of molecular chaperones, a group of proteins involved in assisting not only in the folding of other proteins but also in their degradation. These two processes are carried in most cases by the coordinated functions of different chaperones that form transient complexes, thus forming an assembly line that make more efficient the protein folding and degradation processes. We are currently working with chaperones such as CCT, Hsp70, PFD, and PhLP.

We also study the structure of the centrosome and of some centrosomal complexes and proteins. The centrosome is



Left, Cryoelectron microscopy image of the centrosome. Right, Image of several centrioles.

the major microtubule organising centre (MTOC) in most animal cells. Typically, centrosomes are made of a pair of centrioles embedded in the amorphous, pericentriolar material (PCM). We analyse the overall structure of the centrosome using two approaches, the first one of which is electron tomography, a technique that has recently undergone major improvements, and which might allow the structural characterization of entire centrosomes in near-native conditions. The second approach is X-ray tom-

ography, a technique that certainly allows the reconstruction of whole centrosomes, albeit at lower resolution than electron tomography; we plan to use the facilities being set up in the dedicated beam line at the Spanish ALBA Synchrotron. We are also working on the structural characterisation of centrosomal proteins using conventional electron microscopy.

Finally, we are also interested, in collaboration with the group of Dr. José L. Carrascosa, in the characterisation of the forces involved in the function of certain proteins, and in the manipulation of cell organelles using single-molecule techniques such as optical and magnetic tweezers.

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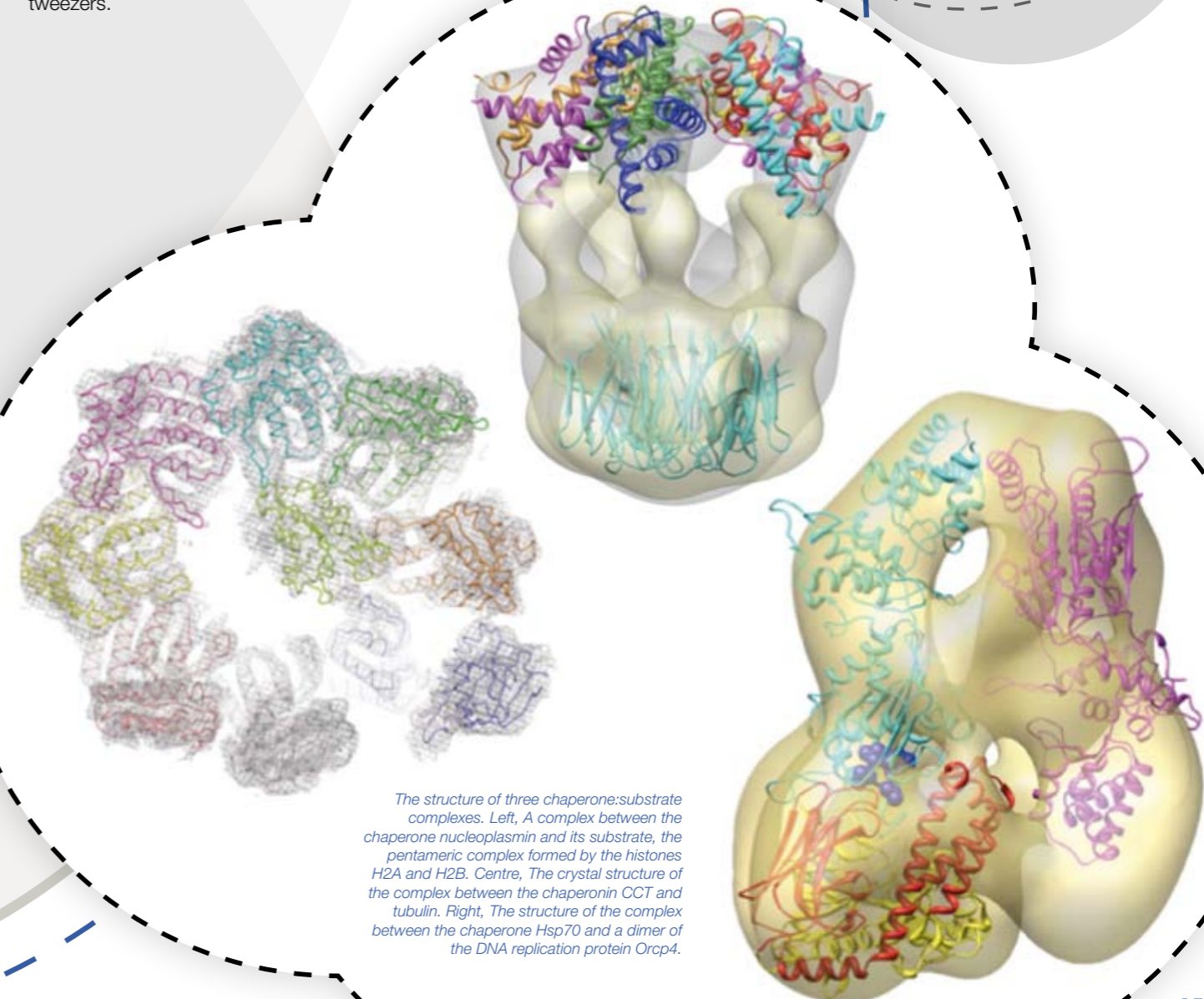
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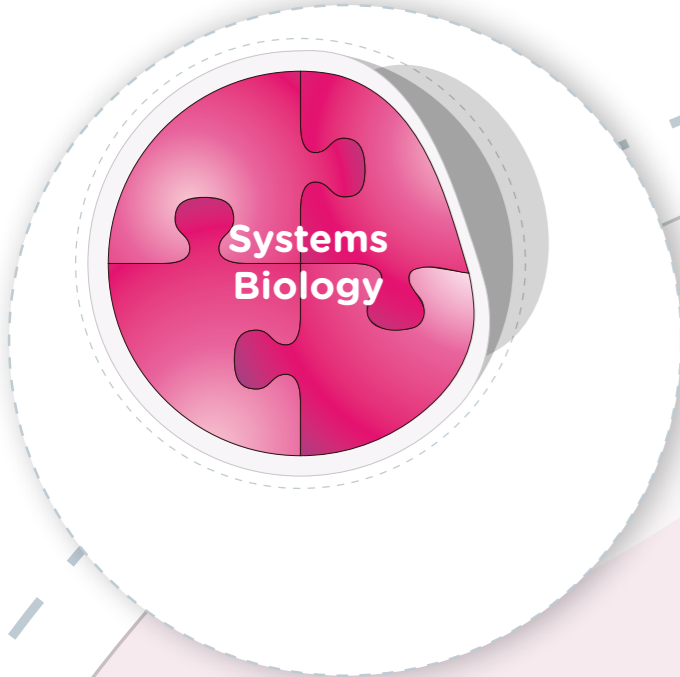
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The structure of three chaperone:substrate complexes. Left, A complex between the chaperone nucleoplasmin and its substrate, the pentameric complex formed by the histones H2A and H2B. Centre, The crystal structure of the complex between the chaperonin CCT and tubulin. Right, The structure of the complex between the chaperone Hsp70 and a dimer of the DNA replication protein Orcp4.



One of the great conceptual novelties of recent years in life sciences research is so-called Systems Biology (and its technological ramification, Synthetic Biology). Systems biology seeks to address the complexity of living systems as such, rather than divide them into smaller parts (unlike the extreme reductionism of traditional molecular biology). The issue is not only understanding, but also modifying and ultimately recreating novel biological systems through the application of a variety of tools and expertise derived from the physical sciences, engineering, biology, computer science and mathematics. Systems biology thus addresses naturally evolved biological systems in a holistic context that considers the emergent properties of layered levels of complexity. On the other hand, synthetic biology tends to focus on the application of the engineering cycle of design, modelling, construction and testing, with a certain emphasis on strong manufacturing concepts including standardization and abstraction. The scientific and technological potential of systems and synthetic biology are immense, in the fields of biomedicine and industrial, agricultural and environmental biotechnology. This has led to the creation of new programmes and centres devoted to the discipline in many of the most respected international research organisations. As a reaction to this challenge, the CNB is leading an ambitious initiative to translate the existing critical mass in these fields into a sound, in-house research area.

VÍCTOR DE LORENZO

Molecular Environmental Microbiology

154

FLORENCIO PAZOS CABALEIRO

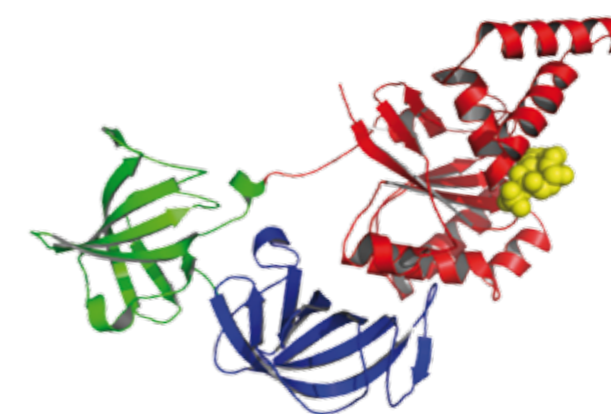
Bioinformática de Sistemas

156

JUAN F. POYATOS

Logic of Genomic Systems

158



Systems Biology

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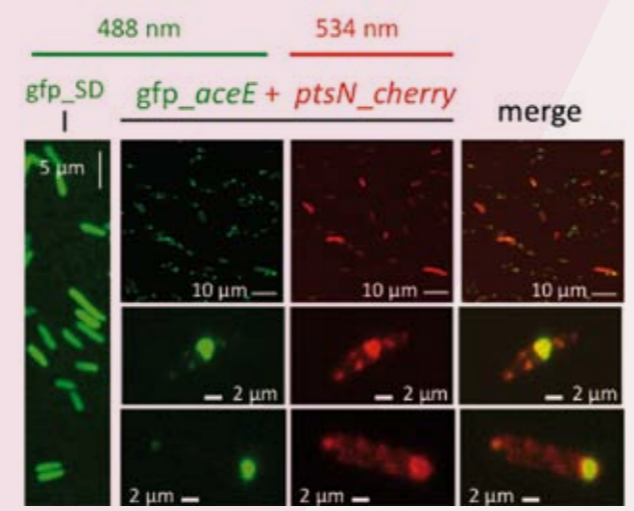
Inés Merino

Molecular Environmental Microbiology

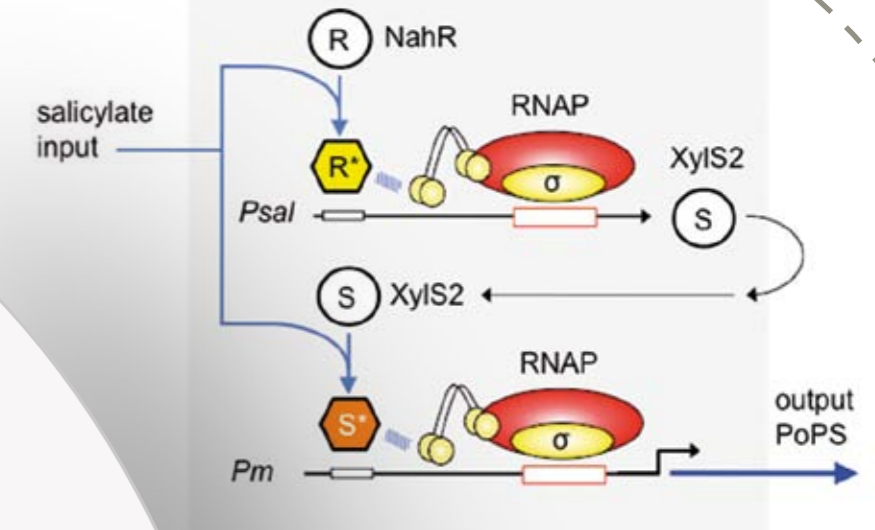
Our laboratory is committed to understanding how bacteria that inhabit natural niches sense and process multiple environmental signals into distinct responses –both at the level of single cells and as a community.

Unlike laboratory settings, in which growth conditions can be controlled and changed one at a time, bacteria in the environment must perpetually make decisions between activating metabolic genes for available, frequently mixed C-sources and those for escaping or adapting to physicochemical stress. Our preferred experimental system involves the strain KT2440 of the soil and plant root colonizer *Pseudomonas putida* bearing the plasmid pWWO, which allows growth on toluene and m-xylene as the only C and energy source. The biotechnological side of this biological question is the possibility of programming bacteria for deliberate environmental release, aimed at biodegradation of toxic pollutants or as biosensors for monitoring the presence of given chemicals. Apart from understanding and developing such sensor or catalytic bacteria, their release requires the GMO to be endowed with a high degree of containment and predictability. In this line, our research takes on board the development of novel molecular tools for the genetic analysis and construction of soil microorganisms (mostly *Pseudomonads*) destined for the environment or as catalysis for selected biotransformations. We recently became active in

the interface between synthetic biology and environmental microbiology as a source of new tools for addressing some outstanding environmental pollution problems. We are currently developing technologies for deep genetic engineering of *P. putida* and other environmental bacteria. These have allowed both a complete understanding of various catalytic and physiological processes and have opened up exciting opportunities for a radical genomic refactoring of the corresponding biological agents. One of the outcomes involved the design of bacteria able to translate the presence of residues of explosives in soil (which can be traced by following the fate of 2,4 dinitrotoluene) into a luminescent signal. At the same time, we studied the logic programs that underlie many of the metabolic and regulatory networks that endow *P. putida* (and other bacteria) with the ability to endure harsh environmental conditions, and found most of them amenable to modelling with Boolean formalisms. Since digital circuits based on logic operations are the basis of computation, we recently began to develop a suite of basic, connectable Boolean gates using regulatory and metabolic components aimed at programming cells to behave in a predetermined way.



Co-localization of PtsN and the pyruvate dehydrogenase AceE subunit in the cytoplasm of *Pseudomonas putida* cells. *P. putida* MAD2 was either transformed with pVLT_gfp_SD or co-transformed with pIZ_gfp_aceE plus pVLTptsN_cherry, expressing respectively red fluorescence-labelled AceE and green-fluorescence labelled PtsN. Cells were grown in LB with 500 μM IPTG to the late exponential growth phase and analyzed in a confocal laser microscope. Fields shown were then excited at 488 nm and 534 nm for emission of green and red fluorescence, respectively, in order to distinguish the production and location of the corresponding proteins. Note the uniform distribution of GFP in the cytoplasm of the control construct (lane 1), while Ace and PtsN (lanes 2 and 3) localize in distinct foci that can be considerably overlapped in the merged images (lane 4).



The NahR/XylS2 feed-forward loop. The scheme shows the first synthetic regulatory cascade for amplification of gene expression reported in the literature. The design included the salicylate-responsive activators NahR and XylS2 from two *Pseudomonas* strains, and their respective cognate promoters PsaI and Pm. Expression of xylS2 was placed under the control of the nahR/PsaI system. When cells face the common effector of the two regulators, both the increase in XylS2 concentration and the stimulation of its activity with salicylate act synergistically on the Pm promoter (having PoPS as its output, see above). This type of regulatory layout could be later classified and formalized as a feed-forward loop (FFL).

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PATENT

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Systems Biology

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Bioinformática de Sistemas

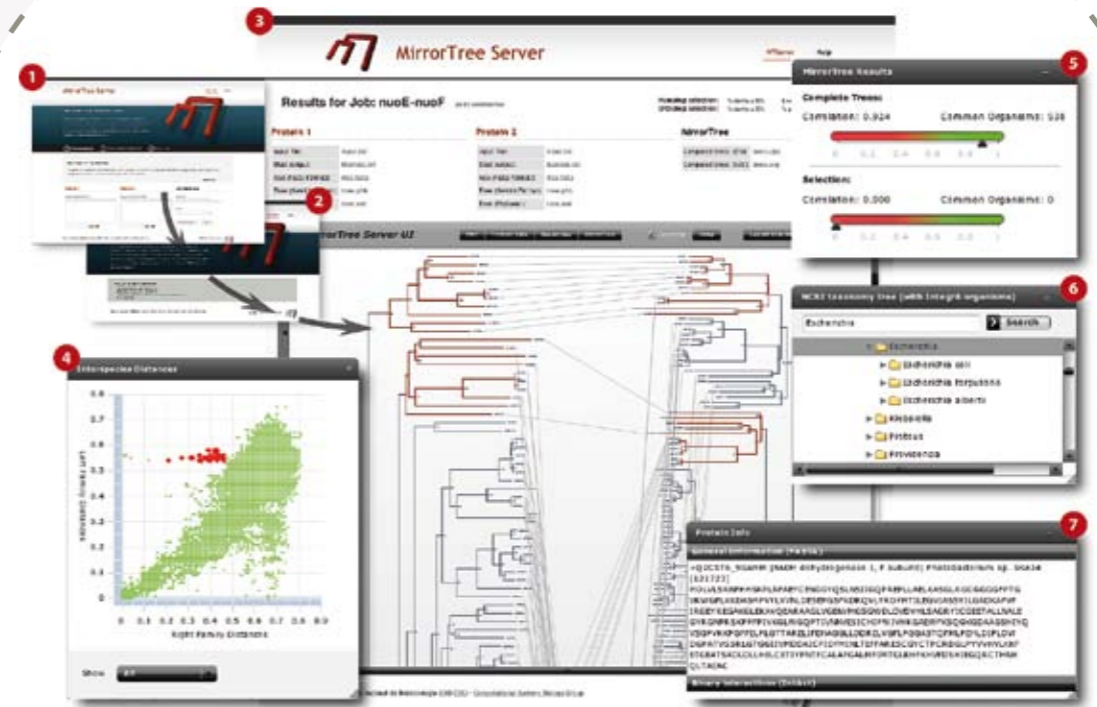
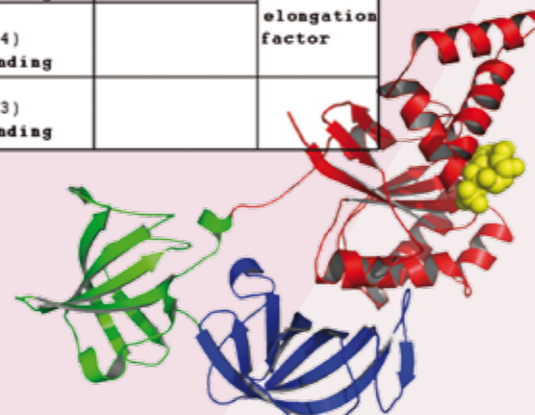
Our group is interested in different aspects of Bioinformatics, Computational Biology and Systems Biology.

Our goal is to obtain new biological knowledge with an “in-silico” approach which complements the “in-vivo” and “in-vitro” methodologies of Biology. This mainly involves mining the massive amounts of information stored in biological databases. Besides our lines of scientific research, we also collaborate with experimental groups providing them with bioinformatics support for their specific needs, and participate in different teaching projects.

We have developed evolutionary-based method for predicting sites with some functional importance in protein sequences and structures. Experimental determination of functional/active sites can not cope with the massive stream of new sequences coming from genome sequencing projects. Hence, computational methods are highly demanded for this task. The methods we develop in this area are based on the fact that functional sites are subject to certain evolutionary constraints whose landmarks can be detected on multiple sequence alignments.

Domain	Pfam/Interpro	scop2go
d1jnyA3	(PF00009) GTP-binding	GTP-binding
d1jnyA1	(PF03144) GTP-binding	elongation factor
d1jnyA2	(PF03143) GTP-binding	

Results of the “scop2go” method for transferring functional annotations to the domain level for the *Sulfolobus solfataricus* elongation factor 1a (PDB: 1jny_A). Comparison with the corresponding annotations inferred from Pfam/Interpro, which incorrectly assign the 3 domains as “GTP-binding”. The GTP bound to the N-terminal (red) domain (SCOP:d1jnyA3) is shown in yellow.



Screenshots of the interface of the MirrorTree server for studying the co-evolution between protein families.

The biological functions of many proteins can only be explained in the context of their relationships with others. Experimental techniques for the determination of interaction partners are still far from perfect and computational methods for predicting pairs of proteins which interact or are functionally associated have emerged. We have developed evolutionary-based methods for predicting interaction partners which have been accepted and followed by the community. These methods are mainly based on the hypothesis that interacting or functionally related proteins adapt to each other during the evolutionary process (co-evolution). We try to detect the landmarks that this co-evolutionary process left in the sequences and structures of the proteins.

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Systems Biology

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Logic of Genomic Systems

Signals continuously impinge on cells, modifying their behaviour.

This can be understood at three levels. At a population level, signals could modify the distribution of cell classes in a population, influencing core cell processes such as cell renewal in stem cell niches. At the intracellular pathway level, signals are effectively sensed and processed by combinations of genetic circuits. An open question is how these circuits distinguish signal from noise, and how circuit structure might limit this ability. Finally, at the signal response level, transcriptional control is fundamental to activate adequate responses. This implies the ability of transcriptional factors to distinguish specific nucleotide sequences in the genome. In the last two years, we have analysed these three aspects of signal processing in the lab.

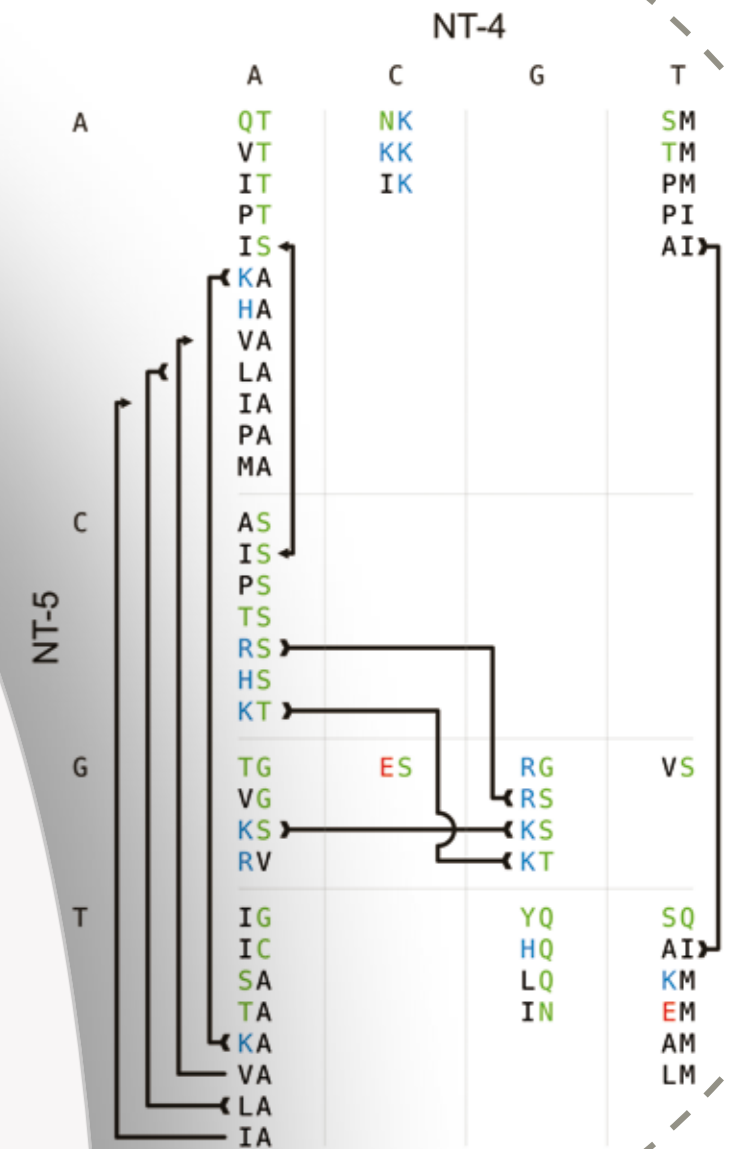
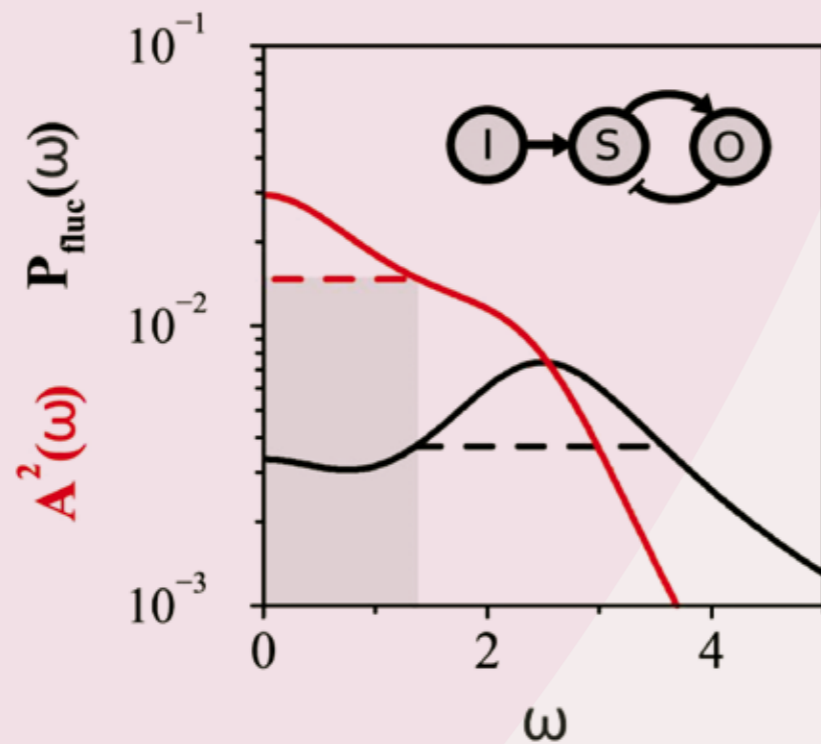
Presence of a wide-coverage recognition code within the LacI family. AA sequences (AA-15, AA-16) recognising a same sequence of NT (NT-5, NT-4) were grouped. Here, we only considered significant palindromic NT sequences. Recognition degeneracies are represented as unidirectional arrows (asymmetrical intrinsic), bidirectional divergent arrows (symmetrical intrinsic), and bidirectional convergent arrows (extrinsic). Polar (green), basic (blue), acidic (red) and hydrophobic (black) amino acids.

1) Signals and populations. Competition for the survival factor Dpp leads to the proliferation of one class of cells (the winners) at the expense of other (loser) cells; both types exhibit normal growth in homotypic environments. Recent studies in *Drosophila* demonstrated the role of the dMyc protein in this process. We examined competition in the (*Drosophila*) ovary stem-cell niche, and showed that differential expression of dMyc triggers competitive interactions. We also presented data in support of the hypothesis that such ongoing competition –between high dMyc stem cells and low dMyc differentiating daughters– increases the efficiency of the differentiation program.

2) Signals and genetic circuits. We considered different types of signals acting on a two-component module to theoretically analyse information processing by genetic circuits. We showed that the presence of feedback in the module imposes a trade-off on amplitude and frequency detection. A direct interaction between the signal and the output species, in a type of feed-forward loop architecture, greatly modifies these trade-offs. Our study emphasised the limits imposed by circuit structure on its stimulus response, and the paradoxical advantage of improving detection with noisy circuit components.

3) Signals and transcriptional control. Transcription factors (TF) commonly act as effectors of cell signal processing by binding DNA sequences adjacent to the response genes whose production they regulate. Could a wide-coverage recognition code between interacting amino acids (of the TF) and nucleotides be found? Our analysis suggested that a set of relatively consistent recognition rules does apply for the extensive LacI family of TF. These rules could ultimately act as a blueprint for the synthetic redesign of TF with new specificities.

Signals can be discriminated from fluctuations when their characteristic frequencies are different from the noise frequency content. In this case, a negative feedback circuit whose fluctuation bandwidth (dashed black line) is always at higher frequencies than the oscillatory bandwidth (red dashed line), allowing for a noise-free frequency regime in transmitted oscillations (grey shaded region).

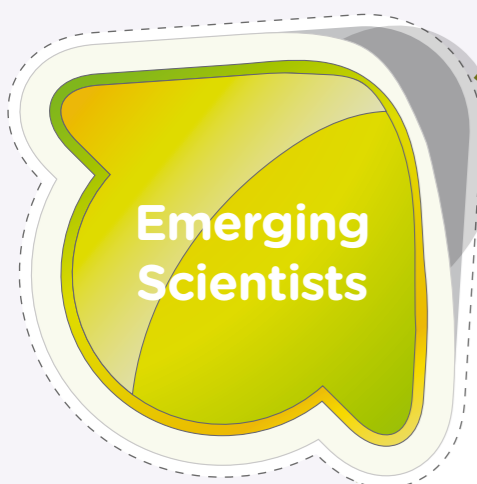


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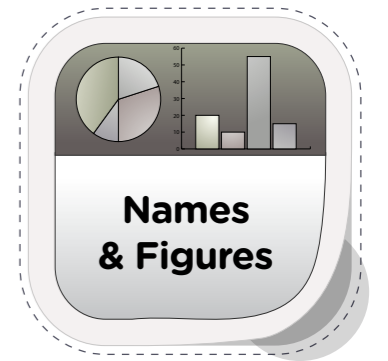
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Inés Antón	162
Sylvia Ayora Hirsch	162
Domingo F. Barber	163
Florencio Pazos	163
Enrique Rojo	164
Carmen Rivas	164
Juan Poyatos	164
Jesús María Salvador	165
Carmen San Martín	165

Doctoral Theses 2009-2010	184
Scientific Meetings	186
Scientific Advisory Board	187
CNB Patent Portfolio	188
CNB Staff 2009-2010	188
Research Projects	189
Scientific Publications	190
Highlighted Journals	191



Scientific & Technical Services

Mouse Embryo Cryopreservation Facility	166
Transgenesis	167
TILLer Service	168
CSIC-PCM Ultrasequencing Platform	168
Bioinformatics Initiative	169
Scientific Computing Service	169
Genomics Unit	170
Sequence Analysis & Structure Prediction	171
Proteomics Facility	171
Confocal Microscopy Service (SMC)	172
Electron Microscopy	173
CNB Histology Facility	173
Flow Cytometry Facility	174
Protein Tools	175
Macromolecular X-Ray Crystallography	175
In vitro Plant Culture	176
Greenhouse Service	177
Animal Facility	177

Radiation Protection & Biological Safety Service	178
Washing & Sterilization	178
Biosafety Level 3 Laboratory	179
DIO Cell Culture	179
Economic Services	180
Project Management	180
DIO Management	180
Information Technologies	181
Human Resources	181

Administrative Services

CNB Library	182
General Services	182
Security	182
Purchasing & Supplies	183
Photography	183
Outreach Manager	183

Emerging Scientists

Inés Antón



In 2003 I was awarded a Ramón y Cajal contract (Centro de Biología Molecular-CSIC-Universidad Autónoma de Madrid) after a two-year stay at the Università degli Studi di Torino and a six-year postdoctoral stay at Children's Hospital (Harvard Medical School) in Boston.

In the laboratory of Prof. Geha I learnt about the primary immunodeficiency Wiskott-Aldrich Syndrome (WAS), caused by mutations in the cytoskeletal and signalling WAS protein (WASP), and WIP (WASP Interacting Protein), a ubiquitously distributed protein that regulates WASP activity and location and stabilizes actin filaments. I generated WIP-deficient mice that have turned into an invaluable tool for the study of actin-dependent functions.

At present, our work focuses on understanding the molecular mechanism that regulates actin polymerization during cellular processes (such as cell adhesion, motility and migration, inflammation, brain and neuronal development, synaptic activity, Golgi architecture and tumor invasion) in different cell types (fibroblasts, dendritic cells, primary neurons, astrocytes and breast cancer cell lines). In 2007 I joined CNB and since 2008 I teach at the Postgraduate Program in Biosciences (Masters in Molecular and Cell Biology, Universidad Autónoma de Madrid).

Sylvia Ayora Hirsch



Sylvia Ayora studied Chemistry at the University of Zaragoza, Zaragoza (Spain) and moved to Germany where she made her PhD in the University of Tübingen, studying microbial extracellular proteases. In 1993 she moved to the Max-Planck Institute of Molecular Genetics of Berlin where she focused on the understanding of DNA repair coupled to transcription in the model system *Bacillus subtilis*.

Upon her return to Spain, she was awarded of a Reintegration Fellowship at the Spanish National Centre of Biotechnology (CNB-CSIC). At the CNB-CSIC she studied DNA replication and recombination in *B. subtilis* and their bacteriophages, followed by a position as a senior researcher ("Ramón y Cajal" and Assistant Professor) at the Universidad Autónoma de Madrid (UAM). Since 2006 she is a CSIC Staff Research Scientist and UAM Honorary Professor.

Her research focuses on understanding recombination-dependent DNA replication and the molecular mechanisms of horizontal gene transfer employing the Gram-positive bacterium *B. subtilis*, and its virus SPP1 as an experimental model.

Domingo F. Barber



Our group is focused in three main topics, all related with the role of lymphocytes in physiological and pathological processes: autoimmune inflammatory diseases, cancer immunotherapy, and nanomedicine.

First, we are trying to dissect the molecular and cellular mechanisms operating in autoimmune diseases. Our goal in this field is to investigate the molecular and cellular mechanisms operating in autoimmune diseases, with the aim of identifying new drug targets and strategies for therapeutic intervention in these diseases.

Second, we are studying the role of NKG2D in autoimmunity and tumour immunotherapy. NKG2D ligands are frequently overexpressed in cancer from multiple origins. In addition, inappropriate expression of NKG2D ligands in cells and tissues make them susceptible to the activation of autoreactive effector cells, therefore triggering or exacerbating autoimmune diseases.

Third, we are developing nanomedicine-based approaches as new therapeutic strategies for treating cancer and autoimmune diseases. Recently, we have shown in mouse models of cancer that IFN- γ -adsorbed DMSA-coated magnetic nanoparticles led to a notable reduction in tumour size. We are in the process of developing and validating a nanoparticle based system for controlled and localized release of small interfering RNAs (siRNAs), microRNAs, and antagomirs for specific gene silencing as a therapeutic application in cancer and autoimmune disorders.

Florencio Pazos



Our group is interested in different aspects of Bioinformatics, Computational Biology and Systems Biology. Our goal is to obtain new biological knowledge with an *in silico* approach which complements the *in vivo* and *in vitro* methodologies of Biology. This mainly involves mining the massive amounts of information stored in biological databases.

Our lines of scientific research can be framed in three main areas: prediction of protein functional and binding sites, prediction of protein interactions, and functional study of biological networks.

Besides these lines we also collaborate with experimental groups providing them with bioinformatics support for their specific needs, and participate in different teaching projects.

Emerging Scientists

Enrique Rojo



I was selected in 2006 to start a group at the CNB as an emerging scientist. Soon after, Dr. Jan Zouhar, a Czech scientist whom I have met during my postdoctoral stay in the US, joined me to develop a project studying vacuolar trafficking in plants. In 2008 the group grew considerably, with the additions of Dr. Michael Sauer, a German scientist, Dr. Alfonso Muñoz, a Spanish scientist, and Otilia Delgadillo, a Mexican PhD student.

The internationality of our group is a reflection of that of the CNB. We have also had great technical support from María Lopez, who was contracted by the CNB to provide support to three emerging scientist groups. The increase in size allowed us to accelerate progress in the vacuolar trafficking project and also to develop a new line of research on the mechanisms that initiate cell differentiation in plants. The CNB has been a great place to initiate my career as an independent researcher, providing an excellent scientific environment and access to most of the equipment and facilities we have needed to develop our projects.

Carmen Rivas



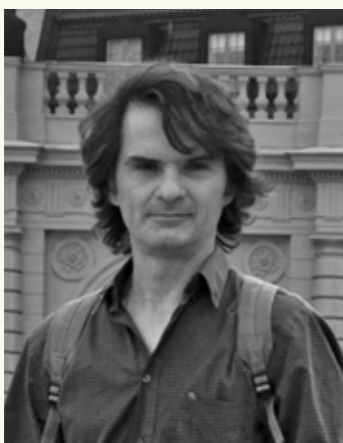
We are interested in the relationship between virus and cancer. We work on two main lines of investigation:

(i) the molecular mechanisms by which virus infection may cause cancer, using the Kaposi's sarcoma associated virus (KSHV) as a model of oncogenic virus and

(ii) to evaluate the importance of tumour suppressors in the complex innate antiviral host defence, and to identify the mechanism by which viruses try to evade the antiviral mechanism imposed by these cellular proteins.

In particular, we are also interested in the role that the cellular proteins, SUMO and ubiquitin, play in these processes.

Juan Poyatos



Modern Biology could be considered as the biggest reverse engineering project ever made, the ultimate task being to identify the function and evolution of the design principles of biological systems. This is the main goal of the Logic of Genomic Systems laboratory led by Juan F. Poyatos.

With a background in theoretical physics, Juan F. Poyatos is now trying to apply an interdisciplinary approach to study how the integration of many molecular elements, as biochemical circuits or networks, ultimately determines cellular function, and whether, and how, this integration is constrained by the intrinsic structure of the genome. These issues are multifaceted in nature, therefore his lab combines several complementary disciplines to address them, such as Systems Biology, Comparative Genomics and Synthetic Biology. Our aim is to understand the function and evolution of the genomic circuitry and also its potential of reprogramming.

Jesús María Salvador



In our group we are dissecting the signalling pathways involved in T cell activation and differentiation to identify novel therapeutic targets in autoimmune diseases and cancer.

T cells are central orchestrators of the cell-mediated immune responses in autoimmune diseases such as rheumatoid arthritis (RA). Antigen-activated T cells stimulate macrophages, monocytes and synovial fibroblasts to produce cytokines which drive inflammation in RA. The p38 MAP kinase (MAPK) regulates critical functions in T cells and it is important in the pathogenic immune response in RA.

We have analyzed p38 activation on T cells from healthy controls and patients with RA or ankylosing spondylitis (AS) to identify variables associated with p38 phosphorylation and disease activity. We found that p38 phosphorylation on Tyr323 was higher in T cells from patients with active RA, than in patients with RA in remission or with AS. Tyr323p38 phosphorylation was associated with disease activity determined by Disease Activity Score (DAS28).

Our results indicate that phosphorylation status on Tyr323p38 correlates with RA disease activity and suggest the Tyr323-dependent pathway as a selective target for downregulation of p38 activity in RA patients.

Carmen San Martín



Carmen San Martín started her career as manager of the Electron Microscopy Facility of the Centro de Biología Molecular "Severo Ochoa" (CBM-SO, CSIC-UAM), while simultaneously working on her M.Sc. degree in Physics (Optics and Structure of the Matter, Universidad Autónoma de Madrid) and her Ph. D. Degree in Physics (Electronics and Computation, Universidad de Santiago de Compostela). She later joined the Wistar Institute in Philadelphia as postdoctoral fellow, funded by a succession of EMBO, HFSP and Spanish Ministry of Education fellowships.

Upon her return to Spain she was awarded a CSIC I3P research scientist position at the Centro Nacional de Biotecnología. Since 2005 she is a CSIC tenured assistant professor, and in 2007 she started her own research group as a CNB junior group leader. She has participated in the development of single particle and EM-Xray combination computational methods, and applied them to the study of replicative helicases and large icosahedral viruses. Her current interests focus on the structural and physical principles that govern assembly and stabilization of complex viruses. Her group approaches the problem from an interdisciplinary point of view, combining Biophysics, Computational, Structural and Molecular Biology techniques.

Research Support

Mouse Embryo Cryopreservation Facility



LEAD INVESTIGATOR

Lluís Montoliu

PERSONNEL

Julia Fernández Punzano
María Jesús del Hierro Sánchez
Óscar Javier Sánchez Sánchez
Marta Castrillo Labrador

The Facility currently offers the following services: freezing 8-cell mouse embryos, freezing IVF-derived 2-cell mouse embryos, thawing mouse embryos and associated embryo transfer procedures to pseudopregnant females for revitalisation of mouse lines, freezing mouse sperm, thawing mouse sperm and in vitro fertilization (IVF), storage of cryopreserved mouse embryos or sperm in liquid nitrogen. A variety of cryopreservation methods and cryopreserving agents are studied and tested to optimise and apply the most up-to-date, efficient protocols. On request, we teach mouse cryopreservation techniques to interested visiting scientists and technicians.

The CNB also hosts the Spanish node of the European project EMMA (European Mouse Mutant Archive, www.emmanet.org), under the coordination of Dr. Lluís Montoliu,

whose objective is the cryopreservation, organised archiving and coordinated distribution of mouse lines of interest to the biomedical scientific community. The current EMMA project, EMMAservice (2009-2012), funded by the European Commission (7th Framework Program), began in January 2009 with a kick-off meeting held at the CNB/CSIC in Madrid (16-17 March 2009), with delegates from all EMMA nodes. Another EMMA meeting dedicated to cryopreservation technology was held at the CNB (18-19 January 2010). EMMA procedures allow the free cryopreservation of mouse lines, and the researcher/submitter need only cover the expenses of shipping mice to the CNB. Researchers can request a grace period of up to 2 years and can include a Material Transfer Agreement associated to the mouse line deposited in EMMA. Distribution of mouse mutant lines cryopreserved at EMMA nodes is associated with the payment of a repository fee. All researchers are given the choice of depositing their mouse mutant lines in EMMA, the first recommended option, or privately within the CNB mouse embryo cryopreservation stocks.

Associated to the EMMA node, the CNB also hosts the Madrid node of the European ESFRI Project INFRAFRONTIER (www.infrafrontier.eu), coordinated by Dr. Lluís Montoliu, which develops infrastructures for large-scale phenotyping and archiving of mouse mutant lines in Europe.

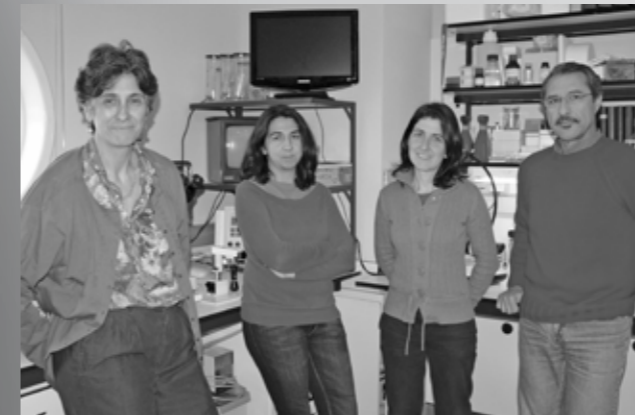
Transgenesis

LEAD INVESTIGATOR

M^a Belén Pintado Sanjuanbenito

PERSONNEL

Alfredo Serrano Montalbo
Marta García Flores
Veronica Dominguez Plaza



Our aim is to determine the molecular bases of the interaction of macromolecules and macromolecular complexes to yield functional biological machineries. We have centred our interest on the way viral proteins assemble into intermediate structures that are further matured to yield fully infective viral particles. Our model systems are bacteriophages (T7 and Φ 29), and eukaryotic viruses such as vaccinia. Using bacteriophages, we have reached subnanometer resolution by electron cryo-microscopy and three-dimensional reconstruction. The combination of these structures with computational modelling has led to the definition of the molecular basis of capsid expansion and stabilization characteristic of certain virus families.

The unit provides the following services:

- Advise in the design of target vectors or constructs for microinjection
- Pronuclear microinjection of plasmidic, BAC or YAC DNA
- Vector electroporation in R1 or G4 ES cell lines
- International consortium ES cells handling and injection to generate chimeras
- Embryo rederivation through IVF or embryo transfer
- DNA purification and founder identification by PCR upon request
- Reproductive biotechnology to solve breeding problems of genetically modified mice.

These activities are combined with training and education on demand and applied research to develop and refine reproductive technologies in order to enhance transgenic production efficiency

Specialized Equipment List:

Two microinjection settings with Narishige micromanipulation system and Eppendorf femtojet injector. Two dissecting microscopes.

- De Fonbrune microforge.
- CO2 incubator and biosecurity hood.
- Pipette puller Termociclator and electrophoresis equipment.



scientific & technical services

Research Support

LEAD INVESTIGATOR

Carlos Alonso Blanco

PERSONNEL

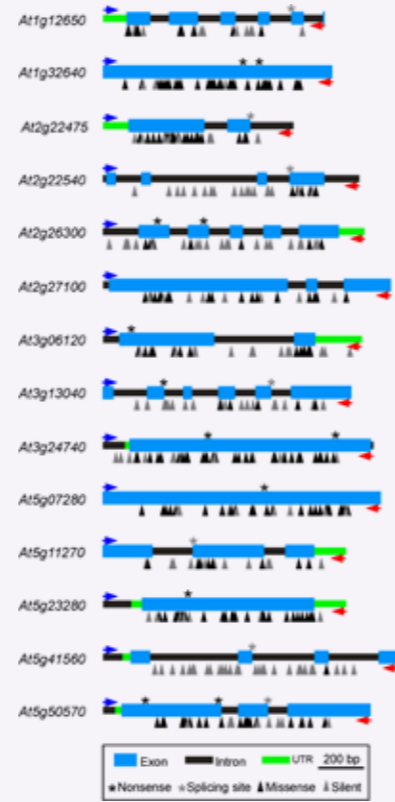
José M. Martínez-Zapater
Beatriz Martín Jouve



TILLer Service is an international public service for the search of EMS induced mutants in the model plant *Arabidopsis thaliana*. TILLer is available through the web page <http://www.cnb.csic.es/~tiller> or through the international Arabidopsis web page (<http://www.Arabidopsis.org/>).

During the past four years TILLer Service has searched for chemically induced mutants by applying TILLING (Targeting Induced Local Lesions in Genomes) technique in an EMS collection of 3712 mutants developed by the service for such purpose (Martin et al., 2009). Up to now, the Service has searched for mutants in more than 20 genes derived from applications from several countries and it has identified more than 500 mutants in those genes.

TILLer Service



14 gene fragments analyzed and mutations found in TILLer collection.

LEAD INVESTIGATOR

José Manuel Franco

CSIC-PCM Ultrasequencing Platform

CNB participates in the CSIC-PCM Ultrasequencing Platform that has recently incorporated two different deep sequencing systems for the analysis of genomes, Genome Analyzer Iix (illumina) and Genome Sequencer FLX (Roche).

Both systems have different but complementary characteristics that guarantee virtually any application involving the analysis of genomes. The number of genomic applications using these systems is almost unlimited, and include the sequencing "de novo" of small and large genomes and massive sequencing of amplicons (specially suitable for the GS FLX system) and all the applications involving resequencing of genomes with the GAllx system, such as sequencing of crop varieties, identification of SNPs for agriculture of diagnostic studies, quantitative and qualitative analysis of transcriptomes including small RNAs, epigenetic studies and the identification of small RNAs and protein-DNA/RNA interactions.

The CNB Genomics Unit also offers computing support for secondary analysis of the results for several applications involving deep sequencing.



Bioinformatics Initiative

LEAD INVESTIGATOR

Alberto Pascual-Montano

PERSONNEL

Juan Carlos Oliveros Alberto Medina
Mónica Chagoyen Quiles José R. Valverde



To improve the visibility and coordination of the existing bioinformatics services at the CNB, the centre has promoted the creation of the Bioinformatics Initiative. The aim of this initiative is to conduct collaborative research and to provide coordinated analysis services to all research groups at the CNB. The Bioinformatics Initiative is composed of four different services:

- Computational Genomics: This service is part of the Genomics Unit and provides bioinformatic support for the analysis, visualisation and interpretation of genomics-related projects, including microarrays and next-generation sequencing.
- Sequence Analysis and Structure Prediction Service: Provides bioinformatic support for sequence and structural prediction topics
- Scientific Computing Service: Covers general

scientific data analysis needs of the CNB and maintenance of the national EMBnet node

- Computational Proteomics: This service is part of the Proteomics Facility and provides interpretation, validation and reporting of data derived from proteomics experiments.

In addition to these services, the Bioinformatics Initiative also includes a set of bioinformatics research groups that can be contacted for collaborative projects:

- Biocomputing Unit
- Functional Bioinformatics
- Image Processing and Statistical Analysis
- Computational Methods for Electron Tomography
- Logic of Genomics Systems Laboratory

Scientific Computing Service

The tasks assumed by the SCS are renewed continuously, adapting to CNB needs. Services are provided using free software tools on CNB and external hardware, and include:

LEAD INVESTIGATOR

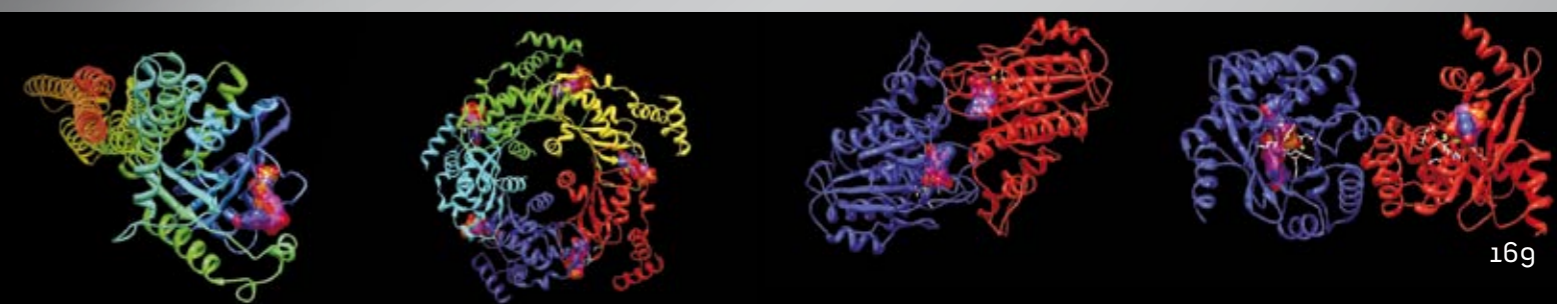
José Ramón Valverde



- Support of advanced scientific computing environments such as clusters and grid at the CNB, CSIC, CESGA and EGEE
- Maintenance of the Spanish EMBnet node:
 - User support and development of data analysis tools and web services
 - Coordination with international bioinformatics networks and institutions
 - Coordination within the CNB to deliver and organize specialized training e-Learning
- Phylogenetic Analysis (comparison, trees, reliability)
- Molecular simulations:
 - Molecular dynamics
 - Docking and macromolecular interactions
 - Computational chemistry (chemical properties, reaction modelling, quantum biology)
- Biostatistics support
- Tertiary analysis of next generation sequencing (NGS) data

The SCS maintains centralised computing infrastructures for all CNB scientists and can provide maintenance and help in supporting group-specific computing infrastructures when needed. Assistance is also offered for experiment and project planning, as well as for customised highly specialized data analysis.

Major recent collaborations include high-throughput genome sequencing, metagenomics, organism classification and OUT-based analysis, analysis of macromolecular interactions of GTP related compounds, coordination of CYTED FreeBIT network, involvement in an NGS data analysis COST action, etc.





Research Support

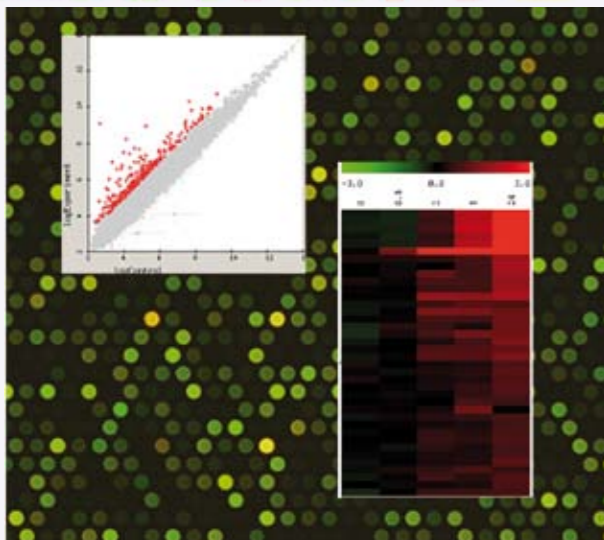
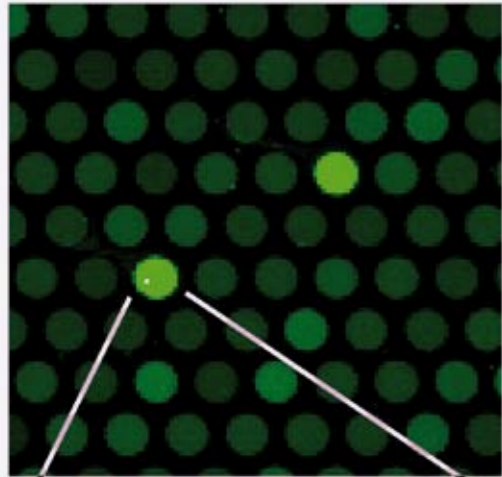
LEAD INVESTIGATOR

José Manuel Franco Zorrilla

PERSONNEL

Gloria García Casado
Irene López-Vidriero
Juan Antonio García
Iria Calvete

Juan Carlos Oliveros
Marta Godoy
Juana María González
Eduardo Gil



Genomics Unit

The Genomics Unit at the CNB focuses on gene expression analysis using microarrays (or DNA “chips”). This technology allows the comparison of gene expression in different biological samples, interrogating the activity of thousands of genes or complete genomes at once, which will contribute to the elucidation of the genetic basis of the biological processes under study. We routinely hybridise and analyse one- and two-channel microarrays. The platforms we support now include Affymetrix, Agilent and NimbleGen, as well as custom microarrays.

We offer services to the CNB and to external researchers, including microarray printing, RNA integrity analysis and microarray hybridisation. We also provide statistical analysis and bioinformatic support. Raw data are analysed statistically using state-of-the-art algorithms, and filtered results are supplied to users in a easy-to-use web-based tool developed by this Unit. We offer advice and support in the use of several bioinformatic tools for functional analysis of genes and genomes, helping users in the biological interpretation of the results.

Research projects are constantly being developed by our personnel, to implement new services and technologies for users. These include new microarray-based technologies such as a new DNA chip for studying DNA-protein interactions, analysis of transcriptome and new strategies for analysis of miRNA-guided degradome.

Equipment

- Complete Affymetrix platform, including fluidics station, hybridisation oven and scanner (3000 7G)
- High-resolution scanner for 1- and 2-colour microarrays (Agilent Microarray Scanner)
- Hybridisation system for NimbleGen microarrays
- Microarray spotter MicroGrid II (Genomic Solutions)
- Bioanalyzer 2100 (Agilent) for analysis of RNA/DNA sample integrity
- Automated liquid-handling workstation (Biomek 2000, Beckman Coulter)
- Laser scanner for two-colour microarrays (Axon 4000B)

Sequence Analysis & Structure Prediction

Sequence analysis and protein structure prediction have proven very useful tools for molecular biology. These approaches, which can be applied in a wide-ranging manner to many areas, can reduce experimental work if well used. Starting from the primary sequence of a gene, these tools can provide data on evolution, functional/structural domain organisation, functionally relevant regions or residues, functional hypotheses, possible subcellular localisation, interactions with other genes, structural models, etc. The main function of the service is to give bioinformatic support for sequence analysis and protein structure prediction, including:

- Localisation of homologous genes within different genomes, totally or partially sequenced, using high-sensitivity methods
- Generation of high quality multiple sequence alignments
- Evolution analysis: gene distribution within organisms/kingdoms, phylogenetic tree generation, etc.
- Subcellular localisation prediction
- Structural/functional domain localisation
- Prediction of functionally relevant residues or regions

- Prediction of post-translational modification sites (phosphorylation, glycosylation, etc.)
- Prediction of interaction sites
- Prediction of functional relationships with other genes by context methods (not homology-based)
- Prediction of physical/functional interactions
- Generation of structural organisation drafts: globular domain localisation, unstructured regions, coiled-coil, transmembrane regions, etc.
- Secondary structure prediction
- 3D structure prediction for close and remote homology
 - Generation of high-quality structural representations for publication

LEAD INVESTIGATOR

Florencio Pazos Cabaleiro

PERSONNEL

Monica Chagoyen Quiles
Juan Carlos Sanchez Ferrero

Proteomics Facility



LEAD INVESTIGATOR

Juan Pablo Albar

PERSONNEL

Alberto Paradela
Silvia Juarez
Marisol Fernandez
Alberto Medina
Rosana Navajas
Sergio Ciordia
Fernando Roncal

The CNB Proteomics Facility (created in 1999) aims to develop a technological platform suitable for large-scale protein identification and characterization, offering its services to the CNB scientific community as well as to external researchers.

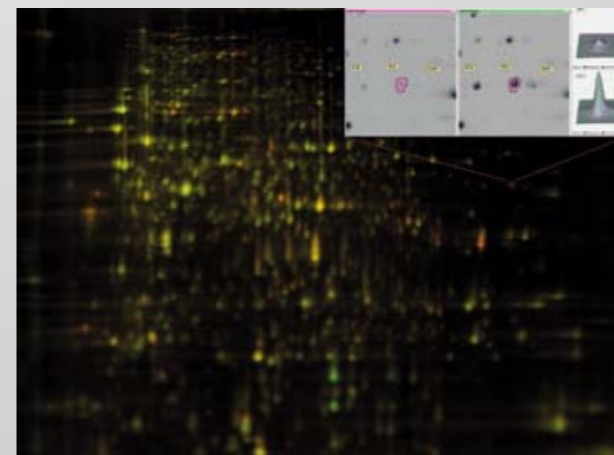
Massive protein identification and characterization is performed through multidimensional nano-HPLC chromatography coupled to either a nano-electrospray ion trap or to a MALDI TOF/TOF mass spectrometer (LC-MS/MS). Comparison between complex proteomes (differential proteomics) is done through the analysis of fluorescent-labeled samples and differential 2D-electrophoresis followed by software-aided sample analysis (2D-DIGE) as well as through the use of isotope stable tags (ICPL, SILAC, iTRAQ) in combination with LC-MS/MS. SELDI-ToF MS (Surface enhanced laser desorption-ionization-time of flight mass spectrometry) is used to obtain protein expression profiles.

For educational purposes, we organize practical courses on topics such as Quantitative Proteomics or bioinformatics. The organization of our lab is described in more detail in: <http://proteo.cnb.csic.es>. Finally, the head of the CNB Proteomics facility (Dr J.P. Albar) is the

General Coordinator of Proteored (National Institute for Proteomics; <http://www.proteored.org>).

Services:

- Two-dimensional electrophoresis/Differential proteomics (2D-DIGE).
- Protein Identification and characterization by MALDI-TOF/TOF, ProteinChip/ SELDI-TOF and/or ESI MS/MS mass spectrometry.
- Protein profiling, purification and biomarker determination by SELDI-TOF MS.
- Identification and characterization of posttranslational modifications.
- Peptide Synthesis and Membrane-bound peptide arrays design.





Confocal Microscopy Service (SMC)

Confocal microscopy imaging techniques provide optical sections of material to observe using lasers and electronic systems of digital image capture. Fluorescent labelling of the sample allows the location of cell components in single sections. In addition, diverse experimental approaches involve single or multiple fluorescent labelling in fixed cells and tissues.

The SMC provides infrastructure for fluorescence, confocal laser scanning microscopy and image processing tools, covering most light microscopy applications, with technical assistance to all users. The equipment and services are available to all CNB personnel as well as to researchers from the public and private sectors.

The technical staff offers formation and advice about equipment use, available methodologies, and for image processing, quantification and later analysis, if required. Aliquots of secondary antibodies and probes with broad use in fluorescence microscopy applications are also provided.

The facility's equipment includes:

- Confocal multispectral Leica TCS SP5 system. Laser lines: 405, 458, 476, 488, 514, 561, 594 and 633 nm, with incubation system for in vivo studies
- BioRad Radiance 2100 confocal system. Laser lines: 457, 476, 488, 514, 543 and 637 nm
- Fluorescence microscope Leica DMI6000B with incubation system for in vivo studies and OrcaR2 monochrome digital camera for image detection
- Two epifluorescence microscopes (Leica DMRXA and Zeiss Axiophot) with colour digital cameras and one Leica stereomicroscope

The unit also provides offline computer workstations for fluorescence and confocal image processing and analysis (LAS AF, MetaMorph, Image J, Laser Pix, Huygens). Auxiliary equipment includes a CO2 incubator, centrifuge, laminar flow chamber, and freezer.

Applications available:

- Laser scanning confocal microscopy
 - Multichannel confocal imaging + transmission imaging of living cells or fixed samples (2D, 3D, 4D imaging)
 - High speed confocal microscopy
 - Multidimensional in vivo time-lapse experiments
 - FRET, FRAP, photoactivation, photoswitching, lambda scan, calcium imaging
 - Subcellular colocalisation studies
- Wide-field microscopy
 - Multichannel fluorescence imaging + transmission imaging (BF, DIC, phase contrast)
 - Multidimensional in vivo time-lapse experiments (wound healing, infection, etc.)
 - Tile scan imaging

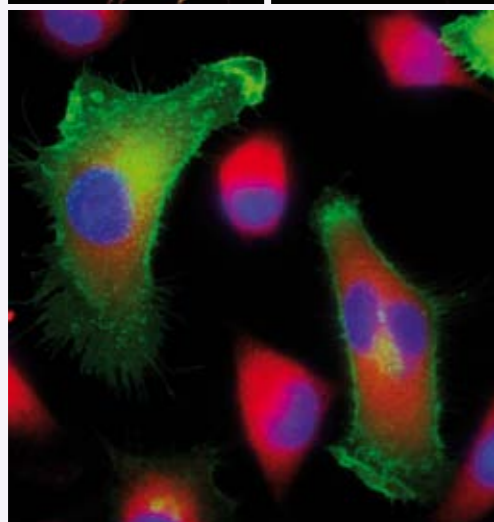
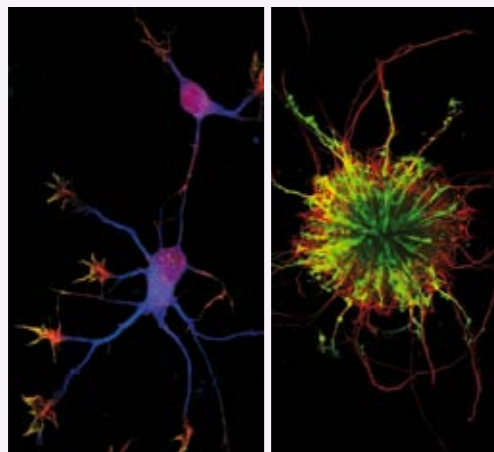


LEAD INVESTIGATOR

Sylvia Gutiérrez Erlandsson

PERSONNEL

Susana Hernández García
Ana Carla García Valenceja



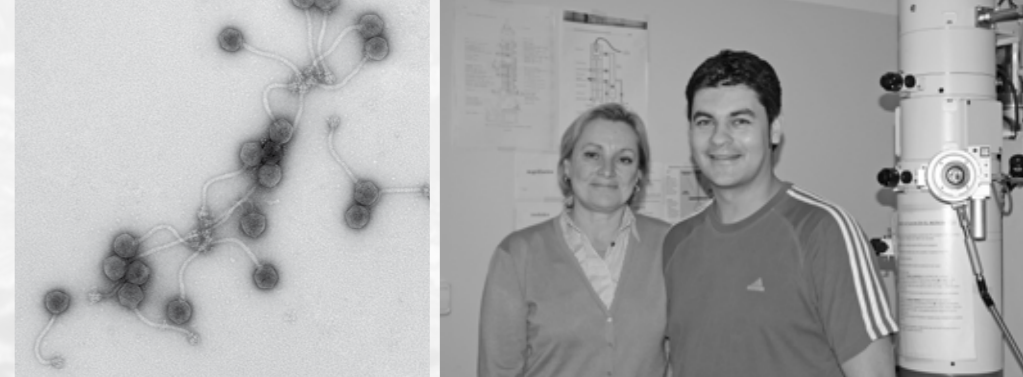
Electron Microscopy

LEAD INVESTIGATOR

Cristina Patiño Martín

PERSONNEL

Javier Bueno Chamorro



The Electron Microscopy service offers a variety of equipment and techniques for the preparation, processing and analysis of biological samples by transmission electron microscopy.

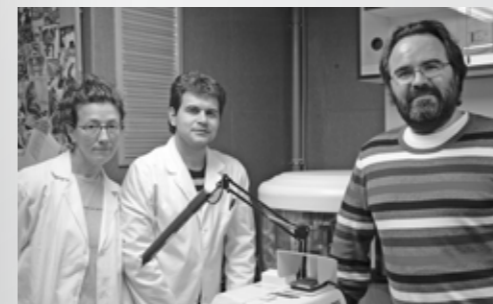
Techniques offered include chemical fixation and inclusion in epoxy and acrylic resins, cryofixation (plunge-freezing, high pressure freezing), cryosubstitution and inclusion in low-temperature resins, ultramicrotomy, immunogold staining, negative staining, negative immunostaining, in situ hybridization, conventional transmission electron and low electron dose microscopy.

The staff provides support to users both in the application of techniques and for equipment use. We offer regular training in the techniques and methods available. We are also responsible, in cases

where required, for sample preparation and image acquisition, and provide support for data interpretation.

The service has the following specialised equipment:

- Jeol JEM-1011 transmission electron microscope with ES1000W
- Gatan camera
- Leica EM MED 020 carbon coating system
- Leica Ultracut UC6 cryo-ultramicrotome with Leica FC6 cryo-chamber
- Reichert Ultracut E ultramicrotome
- Leica EM AFS2 automatic cryosubstitution system
- Leica EM PACT 2 instrument for high-pressure vitrification
- Leica EM TRIM sample trimmer (pyramitome)
- Reichert Knifemaker



CNB Histology Facility

LEAD INVESTIGATOR

Lluís Montoliu

PERSONNEL

Soledad Montalbán Iglesias
Óscar Sánchez Sánchez

At the CNB Histology Facility, we offer methods for the histological analysis of animal and plant biological samples. Available methods include the preparation of paraffin/wax blocks and plastic (HistoResin) for obtaining histological sections with an automated microtome, as well as preparation of blocks to obtain frozen tissue sections with a cryostat. Sections can be counterstained or maintained for later immunohistochemistry analysis. The facility is equipped with a cryostat, two automated microtomes, a tissue processor carousel, paraffin/wax embedding equipment, two water baths, a stereoscope, an oven, and additional small equipment to process all types of tissue samples.

Our expertise is reflected by the wide variety of tissue samples and species we have processed over the past years, including tissue samples from spleen, aorta, femoral, embryo and foetus, brain, cochlea, colon, stomach, ganglia, liver, thin gut, fingers and toes, muscle, eyes, ears, ovary, pancreas, skin, lung, prostate,

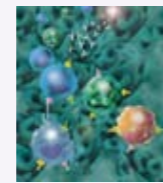
rachis, kidney, brown and white adipose tissue, mammary gland, testis, femur, tibia, gonads, fibula, trachea, thyroid gland, skin and mammary gland tumours, uterus and other tissues from animals including mice, rats, rabbits, sheep, lynx, cat, pig, fish, chicken, gazelle and humans. In addition, we have processed fruits, leaves, apical meristem, roots, stem from tomato, Arabidopsis and potato plants. A full list of tissues processed by the facility is available at: <http://www.cnb.csic.es/~histocnb/tabla.html>.

Since 2009, the CNB Histology Facility is associated with the IIB-UAM/CSIC Histology Facility (www.iib.uam.es/servicios/patexperimen/intro.es.html). The two centres merged their operations under the coordination of the CNB Histology Facility, enabling them to offer CNB and IIB researchers greater processing capacity of histological samples. The CNB Histology Facility has been training personnel from the IIB-UAM/CSIC Histology Facility and supervising work, aiming to establish equivalent output standards.



Flow Cytometry Facility

Flow Cytometry (CMF) is a multiparametric cell analysis technique whose purpose is to detect and identify molecules and cell structures using fluorescent markers and conjugated antibodies. Flow sorting is an extension of this technology, by which any cell or object measured can be separated selectively from the suspension based on properties measured in flow. The facility provides scientific and technological support to the different CNB research groups that require it, for which have the technical and human means suitable to guarantee maximum quality and continuous improvement of all our services.



The facility offers:

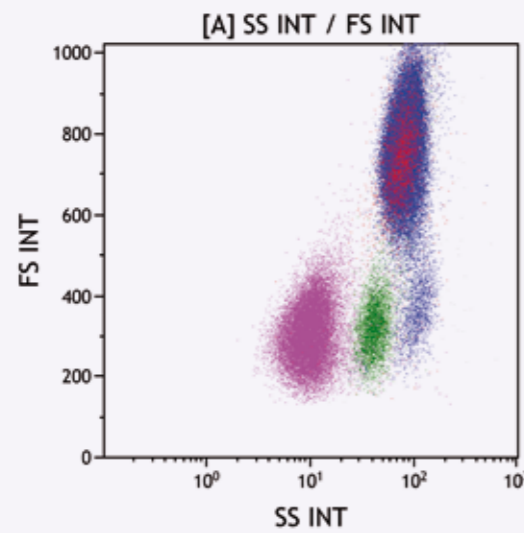
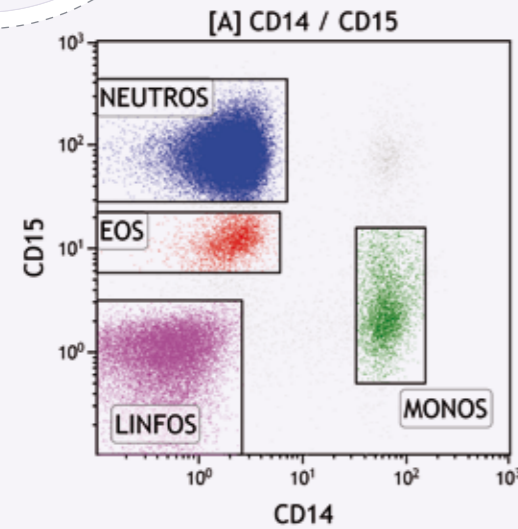
- Training and advice on the principles and applications of analytical flow cytometry, to obtain maximum advantage

- Development and optimization new applications incorporating new technologies and new reagents
- Quantification of secreted cytokines by multiplexed assays
- Result analysis using specialised software packages
- Cell isolation by cell sorting (sorting of cell populations including cell suspensions derived from any animal organ as well as from cell lines)

Equipment:

- BD FACSCalibur analyzer: 4 colours, 2 laser excitation (488 and 633 nm)
- 2 Beckman Coulter EPICS XL-MCL analyzers: 4 colours, 1 laser excitation (488 nm)
- Beckman Coulter Cytomics FC500 analyzer: 5 colours, 2 laser excitation (488, 633 nm)
- BD LSRII analyzer: 8 colors, 3 laser excitation (488, 633 and 405 nm)
- Beckman Coulter Gallios analyzer: 10 colours, 3 laser excitation (488, 633, 405 nm)
- Luminex 100 IS multiparametric analyzer: a system that can be used to quantify multiple cytokines (up to 100) or any other soluble molecule from a single sample
- Cell Sorter Beckman Coulter EPICS-ALTRA Hypersort

The unit also provides the computer science system to analyze the results obtained: 1 Mac and 2 PC platforms running specialised software packages (Cell Quest (Mac), WindMDI (PC), CXP (PC), MultiTime (PC), MultiCycle (PC), DIVA (PC), FlowJo (PC), Summit (PC), Kaluza (PC), etc.

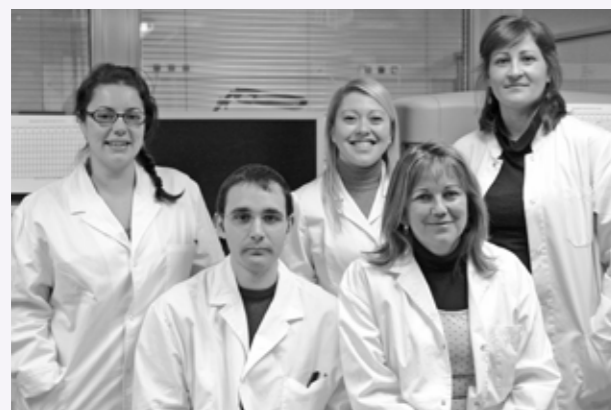


LEAD INVESTIGATOR

M^a del Carmen Moreno-Ortiz Navarro

PERSONNEL

Sara Escudero García
Pablo García Bravo
Sonia Rodríguez Murcia
Almudena González García



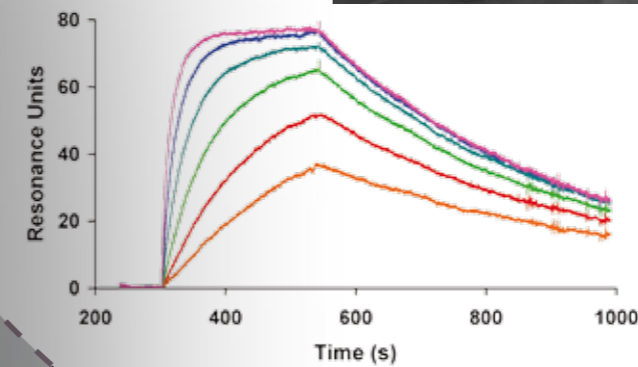
Protein Tools

LEAD INVESTIGATOR

Leonor Kremer

PERSONNEL

María Teresa Martín
Mónica García-Gallo
Mercedes Llorente
Laura Martín
Carolina Sánchez
Lucio Gómez



In summary, the unit produces and characterizes protein tools designed to interact with other molecules, develops assays and analyzes the qualitative and quantitative parameters of biomolecular interactions. We also provide users with antibodies, assays, methodologies and advice.

The macromolecular X-ray crystallography facility provides the following services and techniques:

- Advice and supervision of protein production from cloning to expression in bacterial, yeast and eukaryotic systems
- Support and training for protein purification to obtain crystal-grade protein for crystallisation
- Automated macromolecular crystallisation
- Optimisation of crystallisation conditions, applying standard and in-house techniques
- Crystal mounting, access to synchrotron beam time, X-ray diffraction data collection
- Data processing and structure resolution and analysis

Service equipment:

- Three temperature-controlled crystallisation rooms
- Genesis RSP 150 workstation (Tecan Trading AG) nanodispenser robot
- Rigaku Desktop Minstrel system for automated crystallisation plate visualisation
- CrystalTrak database suite for crystallisation screening and improvement of positive trials



The Protein Tools Unit (PTU) is a facility focused on the design, creation and use of proteins as specific molecular tools. Activities include the generation and characterization of monoclonal antibodies (mAb) for research, diagnostic and therapeutic use, immune response studies, protein labeling, development of specific immunoassays and analysis of molecular interactions.

PTU services are provided to CNB scientists and to external researchers in public or private organizations. The staff offers technical expertise in immunobiology and immunochemistry, including theoretical and practical training courses.

More than 130 mAb to a wide range of antigens have been developed successfully. In the last two years, for example, mAb have been produced to nuclear proteins (Dido), enzymes (β -gal), kinases (p38, PKD, GSK3, ANNK1), membrane proteins (PLL-1), receptors (kidins220), viral antigens (SARS, TEGV), and immunoglobulins (IgE).

Biomolecular interactions involving proteins (chemokines, TCR, antibodies), peptides, oligonucleotides, liposomes and small organic molecules are analyzed using a surface plasmon resonance biosensor Biacore 3000.

Macromolecular X-Ray Crystallography

LEAD INVESTIGATOR

César Santiago



scientific & technical services



scientific & technical services

In vitro Plant Culture

The plants ability for the vegetative multiplication (totipotentiality) can be considered as a fundamental process.

When a culture originated from an explant is established can happen different regeneration and morphogenetical process that produce a new plant

The *in vitro* culture techniques are fundamental for propagation and genetic and biotechnologic plant improvement.

The work sterilization conditions, as well as the climatic medium physical factors: humidity, light (intensity and photoperiod), and temperature are essential in the *in vitro* culture techniques.

Specific plant growth chambers are necessary to preserve and control those factors. These chambers are a fundamental infrastructure for different scientific and technologic objectives like:

- **Explant culture:** adventice organs formation, clonation,...
- **Protoplast culture:** transformation studies, genetic variants production,...
- **Seed culture:** F2 individual segregation studies, plant growth studies using nutrients lacking mediums,...
- **Cell culture:** Maintenance of different plant species cell lines.

Services offered

The CNB *in vitro* Plant Culture Service maintains different equipments and offer the necessary techniques for:

- The sow and growth of plant cells, tissues and seeds.
- Their maintenance and propagation.

And so for the obtaining of genetically transformed plant cells by means of

- Permanent plant transformation: using the *Agrobacterium tumefaciens* microorganism (in tomato, tobacco, *arabidopsis*).
- Transitory transformation: using the "Particle Delivery System".

Instrumentation

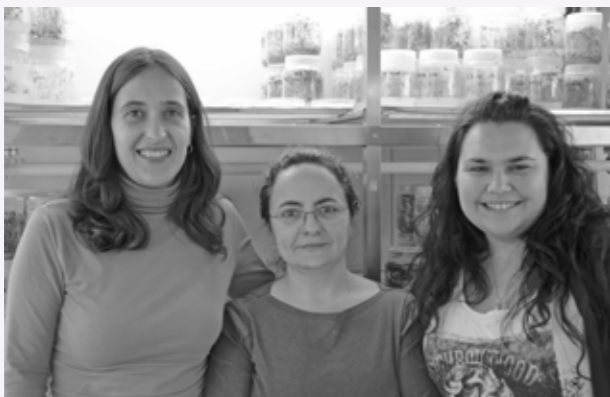
- Laminar flow hoods
- Plant Growth Chambers
- Centrifugal
- Particle Delivery System
- Microscope
- Incubator
- Ovens
- Sterilizer

HEAD OF SERVICE

Raquel Piqueras Martín

PERSONNEL

María Luisa Peinado Vallejo
Beatriz Casal López



Greenhouse Service

HEAD OF SERVICE

Tomás Heras Gamo
Carlos Alonso Blanco

PERSONNEL

Alejandro Barrasa Fustes
Esperanza Parrilla Carrillo
Raúl Pedraza León



The Greenhouse Service takes care of the following facilities specific for plant cultivation:

- A standard greenhouse with 8 cabinets (total growth surface: 180 m²)
- A P2 safety level greenhouse with 4 cabinets (total growth surface: 83 m²)
- 18 climate chambers

The Greenhouse Service carries out the following tasks:

- Growth and propagation of plants under controlled environmental conditions
- Growth and propagation of mutant and transgenic lines under controlled environmental conditions
- Identification, selection and phenotypic analysis of mutant and transgenic plants

Animal Facility

SENIOR TECHNICIANS

Alberto García
Antonio Morale
Mercedes de la Cueva

Javier Martín
Silvia Alves

HEAD OF SERVICE

Ángel Fernando Naranjo Pino

PERSONNEL

Ángel Moreno Calle
Sergio Magallón
Raquel Gutiérrez

Isabel Martín Dorado Caballero
Evaristo Criado
Lucio Gómez



The CNB Animal Facility dedicates its efforts to the generation and maintenance of animals for research. We support scientific personnel in developing new research techniques for their *in vivo* studies. Although our main work is with knockout, knock-in and transgenic mice, we also maintain rats, guinea pigs and zebrafish.

The facility is divided into four sections, based on the microbiological status of each animal colony. Last year, we incorporated new equipment and ventilation racks to increase the microbiological safety of our animals. We have an independent TII section for biological security and a unit for zebrafish husbandry and breeding.

Our personnel participate in the training of researchers in a national course on management and genetics of mouse colonies.



Radiation Protection & Biological Safety Service

The service provides scientific services, training, and guidance on all facets of radiation protection and biological safety at the CNB.

Functions and services:

- Risk assessment
- Acquisition of security and personal protective equipment
- Design of laboratories and installations
- Acquisition and management of radioisotopes
- Edition of internal health and safety manuals
- Processing of regulatory compliance documentation for implementation actions and normal operating conditions
- Training of and information to personnel who work with hazardous material
- Classification and labelling of laboratories
- Control of compliance with health and safety standards, operation and use of installations
- Control of personnel, management of medical surveillance and personal dosimetry, medical and dosimetric record-keeping
- Intervention in accidents and emergencies
- Control of production and packaging of hazardous waste
- Internal transport and storage of hazardous waste before transfer to authorised or controlled disposal facilities

Installations:

The service monitors risk operations in CNB laboratories and directly manages the biosafety level 3 laboratory, the gamma irradiator and the central radioisotope laboratory, which has:

- Two radioisotope cabinets
- Biosafety cabinet
- CO2 incubator
- Ultracentrifuge, centrifuge and microfuge
- SpeedVac
- Hybridisation oven



HEAD OF SERVICE

Fernando José Usera Mena

PERSONNEL

Sonia Calvo Ladrero
Jessica Gaspar Navarro
Aranzazu de la Encina Valencia



Washing & sterilization



HEAD OF SERVICE

Rosa María Bravo

PERSONNEL

Carlos Enríquez
María Trinidad Mba
Ana Isabel Nieto
Josefa Pérez Alfaro
Rosi Ramos
Arancha Rodríguez
Anunciación Romero González
María Ángeles Sánchez



Biosafety Level 3 Laboratory

HEAD OF SERVICE

Fernando José Usera Mena

PERSONNEL

Sonia Calvo Ladrero
Jessica Gaspar Navarro
Aranzazu de la Encina Valencia

The service manages the level 3 biological containment laboratory, consisting of three sublaboratories for in Vitro culture with all necessary equipment for safe handling of biological agents in Risk Group 3 and for type 3 confined activities with genetically modified organisms.

We have following equipment:

- Three biological safety cabinets (BSC)
- Two incubators for animal cell culture
- One incubator for bacterial culture
- One double-door autoclave
- SAS for biological inactivation (small materials)
- SAS for biological inactivation (large materials)
- Refrigerated ultracentrifuge
- Three refrigerated benchtop centrifuges
- Three refrigerated microcentrifuges
- Three inverted optical microscopes
- Liquid nitrogen tank
- Three ultra-low temperature freezers (-80°C)
- Communication systems: computer network and telephone
- Various alarm systems in case of malfunction, incident or accident

DIO Cell Culture

HEAD OF SERVICE

Remedios Proenza

PERSONNEL

Ana Montero
Gema Alcón





Economic Services

HEAD OF SERVICE

Mariano Muñoz Jiménez

PERSONNEL

María José Gregorio Usanos
Carmen Berreiros Cano
M^a Ángeles Lumbreras Carrasco
Elena Barreda García
Irene Vera Jiménez

María Virginia Hortal Robles
Esther Ruiz de la Hermosa Romero
Rafael López Laso
Diana Pastor Calero
Laura Rojas Sanz



Project Management

HEAD OF SERVICE

Soraya Olmedilla María

PERSONNEL

Aurora Cabrerizo Alonso
Pilar Ara Laúna
Daniel Martín Hernando
Pablo Antonio Coret Francés

DIO Management

HEAD OF SERVICE

Antonio Rodríguez Terán

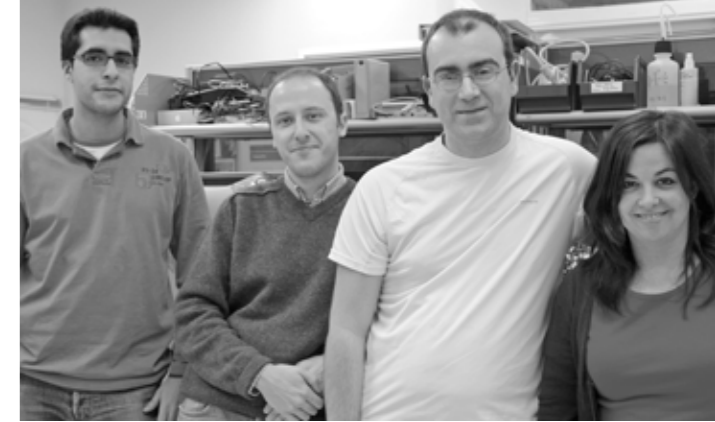
PERSONNEL

Coral Bastos
Catherine Mark

Information Technologies

Among other functions, the service provides:

- Microinformatic support
- Registry of equipment on the network
- E-mail accounts
- Management of distribution lists
- Management of network infrastructure (cable and wireless)
- Shared archive servers
- Information systems security
- Remote access
- Web server (not contents)
- Registry of DNS equipment
- Internet protocol telephony (shared with General Services)
- Server backups



HEAD OF SERVICE

Sonia de Diego

PERSONNEL

Óscar Bodas
Iñigo Oficialdegui
Alberto Sánchez
Patrick Moutel
Jorge Calvo
Antonio Millán

Human Resources

HEAD OF SERVICE

Marina Hernando Bellido

PERSONNEL

María Jesús Raboso Pérez
Susana Pena González
Pablo Antonio Coret Frances
Javier Tortosa Nieto
M^a Jesús Torrado Macías

Procedures:

- Monitoring and control of all grant calls, production of reports, annual reports, final reports etc.
- Formal contractual complaints and preparation of compensations
- Handling of resignations and departures
- Registration, termination and variations in Social Security of contracted staff, civil servants and fellowship holders at the CNB, Institute of Theoretical Physics and the Enological Institute (ICV)
- Registration, termination and variations in accident and medical insurance
- Management, control and processing of all types of leave, holidays, and authorised absences for all staff at the centre
- Management, control and processing applications for service recognition of civil service and contracted personnel
- Management, control and processing of overtime for permanent workforce staff
- Provision of civil service positions through competitive examinations and merit selections
- Management, modification, and control of job descriptions for civil service and workforce staff



- Management and processing of foreign employee contracts
- Management of expenditure for staff and research projects, with maintenance and updating of the database created for this purpose
- Management, processing and control of the internal promotion process for civil servants and workforce staff
- Management of calls for temporary positions
- Management of applications for retirement, extension of service, recognition of seniority, recognition of personal level
- Management and processing of stays abroad of scientific staff (sabbaticals)
- Management, processing and control of different types of productivity allocations
- Certification of documents for selection processes and for project justification
- Preparation of identification cards for access to the various facilities in the centre
- Daily maintenance of the centre's database and the CSIC institutional database for personnel and organizational management
- Preparation of legal and compliance reports
- Support and advice to all staff of the centre, as well as at the Theoretical Physics Institute and Enological Institute, as well as information on all new regulations and personnel management



Research Support

General Services

HEAD OF SERVICE

Gabriel Sánchez de Lamadrid

PERSONNEL

Julián Miguel Grande
Antonio Rojo
Manuel Grande
Ángel Godoy
Vicente Martín
Gema Alcón

Pilar Cutillas
M^a Socorro Muñoz
Lourdes Sánchez
Celia García
M^a Ángeles González
M^a Sol Aguirre
Ana María Puerto
Juan Pablo Illescas
M^a del Carmen García
Eloína Rodríguez
Rosa María Martínez

J. Miguel de la Hoz
Juan José Fontela
Paloma González
Ignacio Santidrian
Carolina Nogales
Calixta Manzueta
Sarah Lenoan
Aillin Notario
M^a Concepción Gómez
Angélica Chicaiza

CNB Library

HEAD OF SERVICE

M^a Dolores Aparicio Trujillo

The CNB Library carries out the following activities:

- Acquisition, technical processing, cataloguing and classification of monographs

(207 monographs acquired and catalogued in 2009 and 2010)

- In-house lending to personnel and laboratories

Loans in 2009: 154

Loans in 2010: 86

- Interlibrary loan services

Requests attended in 2009: 359

Requests attended in 2010: 402

- Access to online databases, e-journals and e-books



Security

HEAD OF SERVICE

Sócrates
Gutiérrez Monreal

PERSONNEL

Jesús Payán Aparicio
José Fernando Albarran Aparicio
Juan José Bech Afonso
Tomás Castro
Yolanda Cantosro

Purchasing & Supplies

HEAD OF SERVICE

Ramón Serrano Coronado

PERSONNEL

M^a Soledad Notario Torres
Montserrat Nerín Toboso
M^a José Caballero Martín
Julio Díez Álvarez
Héctor Hernández Redondo
Antonio Pastor Encabo
Juan Carlos Bermudo Zamora



Photography

HEAD OF SERVICE

Inés Poveda



Outreach Management

HEAD OF SERVICE

Alfonso Mora

The CNB hosts an outreach office which helps our scientists in the dissemination of their results to other scientists and the society.





Doctoral Theses 2009-2010

Esther Rincón Gila

Caracterización de la interacción entre DGK ζ y SNX27: nuevos componentes del tráfico vesicular en linfocitos T

Isabel Mérida San Román

Amit Kumar

Involvement of class IA phosphoinositide 3-kinase (PI3K) in cell division and DNA repair

Ana Clara Carrera

María López-Bravo

Diferenciación y especialización de las células dendríticas de ratón para la inducción de inmunidad innata y adaptativa

Carlos Fernández-Ardavin

Teresa Domínguez López

Modificación genética de las omega-3 desaturasas para mejora del aroma del tomate y estudio de la función de las proteínas fosfatasa PP2Ac en *Arabidopsis*

José Juan Sánchez Serrano & Maite Sanmartín Artiñano

Nerea Irigoyen Vergara

Analysis of the assembly and maturation processes of the Infectious Bursal Disease Virus

José Francisco Rodríguez & José Ruiz Castón

Mireia Vallespinos Serrano

Estudio de la función del proto-oncogén c-myc en la diferenciación de los linfocitos B

Ignacio Moreno de Alborán

Ariel Rodríguez

Acoplamiento funcional entre el virus de la gripe y la maquinaria de transcripción celular. Relación con la patogenicidad viral

Amelia Nieto Martín

Alicia González Martín

Función de CCR5 y sus ligandos en la respuesta del sistema inmune frente a tumores

Santos Mañes Brotón & Emilia Mira Damaso

Pedro Melgar Rojas

Función de la vía de señalización Notch en el desarrollo del sistema hematopoyético

José Luis de la Pompa

Elodie Sartorel

Characterisation of the RAM pathway in *Ustilago maydis*

José Pérez-Martín

Rocío Coloma Ciudad

Análisis funcional y estructural de la RNP recombinante del virus de la gripe

Juan Ortín Montón & Jaime Martín Benito

David Páez

Funcionalidad biológica de la respuesta a arsénico en la bacteria hipotolerante *Pseudomonas putida* KT2440

Víctor de Lorenzo

Carlos Álvarez

Genetic traps and the interaction between effector molecules and transcriptional regulators

Víctor de Lorenzo

Fátima García Quintanilla

Estudio de estructura-función del regulador de crecimiento intracelular IgaA de *Salmonella enterica*

Francisco García del Portillo

Alicia Fajardo Lubián

Búsqueda de genes implicados simultáneamente en la resistencia a los antibióticos y la virulencia de *Pseudomonas aeruginosa*

José Luis Martínez

Elvira Marín Muñoz

Comparative study of the proteobacteria type V secretion system transporter domains and their application for the surface display of antibodies in *E. coli*

Luis Ángel Fernández Herrero

María José Rodríguez Gómez

Caracterización bioquímica y funcional del enzima alfa-dioxigenasa

Carmen Castresana & Gerard Bannenberg

Virginia Lioy

El sistema toxina-antitoxina $\epsilon\zeta$ como inhibidor de la proliferación celular e inductor de tolerancia

Juan Carlos Alonso Navarro

Roberto Melero del Río

Estudio de la estructura cuaternaria de p53 y el complejo p53/ADN mediante microscopía electrónica

José María Carazo & Mikel Valle

Vincenzo Calvanese

Epigenetic regulation of developmental genes in embryonic stem cell differentiation

Mario Fernández Fraga

Silvia Hormeño Torres

Biofísica de centrosomas y ADN mediante manipulación óptica

Ricardo Arias-González & José María Valpuesta

Tomaso Benedet

DREAM differently regulates spinal and trigeminal nociception

José Ramón Naranjo

Domingo Miranzo Navarro

Aislamiento y caracterización del gen *pcsB*, responsable de la conversión de pimaricina en su derivado carboxiamida AB-400

Francisco Malpartida Romero

Álvaro Hernández Fernández

Elementos de resistencia a los antibióticos codificados en el cromosoma de *Stenotrophomonas maltophilia*

José Luis Martínez Menéndez

Patricia Resa Infante

Análisis estructural y funcional de la polimerasa del virus de la gripe

Juan Ortín

Tamara Velloso Armengol

Análisis funcional de oxilipinas: una familia de derivados lipídicos implicados en procesos de defensa contra patógenos y de desarrollo vegetal

Carmen Castresana Fernández

M^a Ángeles Cobos Fernández

Estudio de la regulación de la localización subcelular de diacilglicerol quinasa zeta y su función en el núcleo

Isabel Mérida San Román

Emilio Yáñez López-Cano

Traducción de proteínas en la célula infectada por el virus de la gripe: análisis del requerimiento de factores del complejo eIF4F y de la participación de la polimerasa viral

Amelia Nieto Martín

Miguel de Lucas Torres

Regulación hormonal de la respuesta a la luz en *Arabidopsis thaliana*

Salomé Prat

Marta Rajkiewicz

Structural studies on *E. coli* DNA polymerase III clamp loader subcomplexes

José María Carazo & Carmen San Martín Pastrana

Álvaro Peña Conde

Caracterización estructural y funcional del complejo THO de *Saccharomyces cerevisiae*

José María Valpuesta & José L. Carrascosa

Adrian Valli

RNA silencing suppression, a novel function for P1 serine protease of viruses of the family *Potyviridae*

Juan Antonio García Álvarez

Isabel Mateos Moreno

Análisis genético y molecular del gen *bHLH149* en la respuesta al ayuno de fosfato

Javier Paz-Ares & José Manuel Franco Zorrilla

Paula Cárdenas

Role of polynucleotide phosphorylase during double-strand recognition in *B. subtilis*

Juan Carlos Alonso

Ana Blanco Toribio

Direct injection of functional single domain antibodies into human cells employing the type III secretion system of *Escherichia coli*

Luis Ángel Fernández

Pilar Domínguez Rodríguez

Regulación por lovastatina de la activación de las células dendríticas inflamatorias

Carlos Fernández-Ardavin

Inmaculada Bañón Rodríguez

Implicación de WIP en procesos migratorios: quimiotaxis y migración transendotelial

Inés Antón

Daniel Rozas Saez

Identificación y caracterización funcional del sistema de dos componentes SCO5784-SCO5785 de *Streptomyces coelicolor*, que provoca "Stringent response", regula la síntesis de proteínas extracelulares y altera la producción de antibióticos

Rafael Pérez Mellado

María Almena Carrasco

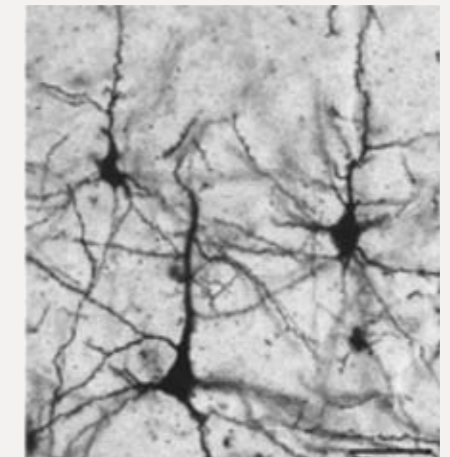
Función del diacilglicerol en el desarrollo, activación y homeostasis de las células T

Isabel Mérida San Román

Manuel Tardaguila Sancho

Circuitos celulares y moleculares regulados por cxc11 en el cáncer de mama y la neurodegeneración

Santos Mañes Brotón





Scientific Meetings

19–21 October 2009
Replication and recombination of RNA virus genomes
Luis Enjuanes

19–23 October 2009
Técnicas inmunológicas: caracterización y cuantificación de proteínas mediante anticuerpos
Leonor Kremer

1–3 December 2009
Fostering Systems & Synthetic Biology in Southern Europe
Victor de Lorenzo

16–17 December 2009
XVI Jornadas Científicas del CNB
Alfonso Mora

28 December 2009
Avances en Biología Molecular por Jóvenes Investigadores en el Extranjero
Domingo F. Barber, Lourdes Planelles, Yolanda R. Carrasco & Alfonso Mora

4 March 2010
Madrid Bioemprende
Fernando Alfaro

6 April 2010
Imagen *in vivo* por fluorescencia y luminiscencia
Beatrice David

21 June– 2 July 2010
Analysis of macromolecular structures

27–30 September 2010
Genética y gestión de colonias de animales de experimentación
Ángel Naranjo

16 October 2010
Jornada ALBA-CIBERER informativa sobre albinismo
Lluís Montoliu & Mónica Puerto

18–19 October 2010
Final COMBACT Workshop
Miguel Vicente

22 October 2010
Regulation of bacterial metabolism
Fernando Rojo

28 October 2010
International Symposium of Vessels and Blood Cells
Luis Álvarez-Vallina, Luis Ángel Fernández & Antonio Rodríguez

3 November 2010
Posibilidades de la espectrometría de masas en el estudio de rutas metabólicas y descubrimiento de biomarcadores
Carlos Pérez

12 November 2010
Inaugural Colloquium of the Systems & Synthetic Biology Program
Rafael Rodrigo, José María Valpuesta & Víctor de Lorenzo

3 December 2010
Fast and versatile isolation of highly purified functional immune cells
Vicky Stronge

20–21 December 2010
XVIII Scientific Workshop
Alfonso Mora

22 December 2010
Avances en Biología Molecular por Jóvenes Investigadores en el Extranjero
Domingo F. Barber, Lourdes Planelles, Yolanda R. Carrasco & Alfonso Mora

At the CNB, we host meetings and seminars for the exchange of scientific results and knowledge. During the years 2009 and 2010, a total of 306 seminars were held, with speakers from outstanding national and international institutions.

To bring science to society, more than 520 high school students visited the CNB in 20 different activities such as Open House Days, practical demonstrations, exhibits and conferences.

We also held various workshops, meetings and courses organised with the collaboration of CNB researchers:

16–17 March 2009
1st EMMA service meeting
Lluís Montoliu

21–24 April 2009
Curso de Proteómica Cuantitativa
Juan Pablo Albar

23 March 2009
Jornada científica sobre celiacía y gluten
Juan Pablo Albar

28 April 2009
Journal Club
Alfonso Mora

7–8 May 2009
1st Workshop on Nanobioscience
José María Valpuesta

25 May 2009
ProteoRed Annual Meeting
Juan Pablo Albar

1 June 2009
Herpesvirus y terapia génica

29 September 2009
Nuevas Herramientas para Combatir Enfermedades Infecciosas
Miguel Vicente

1–2 October 2009
Madrid Meeting on Dendritic Cells and Macrophages
Carlos Ardavin

5–8 October 2009
Genética y gestión de colonias de animales de experimentación
Ángel Naranjo

Scientific Advisory Board

The Scientific Advisory Board, consisting of internationally recognized scientists, discusses with CNB researchers the quality, significance and main focus of the research conducted at the CNB.



Wolfgang P. Baumeister
Electron tomography, proteomics, proteasome structures and function
Department of Molecular Structural Biology - Max-Planck-Institut für Biochemie, Martinsried, Germany.



Maarten Koornneef
Plant breeding, genetics
Plant Breeding and Genetics - Max Planck Institute for Plant Breeding, Cologne, Germany.



Juan Luis Ramos
Bacterial genetics and molecular biology, biorremediation, microbial ecology
Departamento de Protección Ambiental - Estación Experimental del Zaidín, Granada, Spain.



Anne Ridley
Cell signalling, cell migration in cancer and metastasis
Randall Division of Cell and Molecular Biophysics - King's College, London, UK.



Anna Tramontano
Genome functional annotation, protein structure prediction, protein design, viral proteins
Department of Physics - University of Rome "La Sapienza", Rome, Italy.

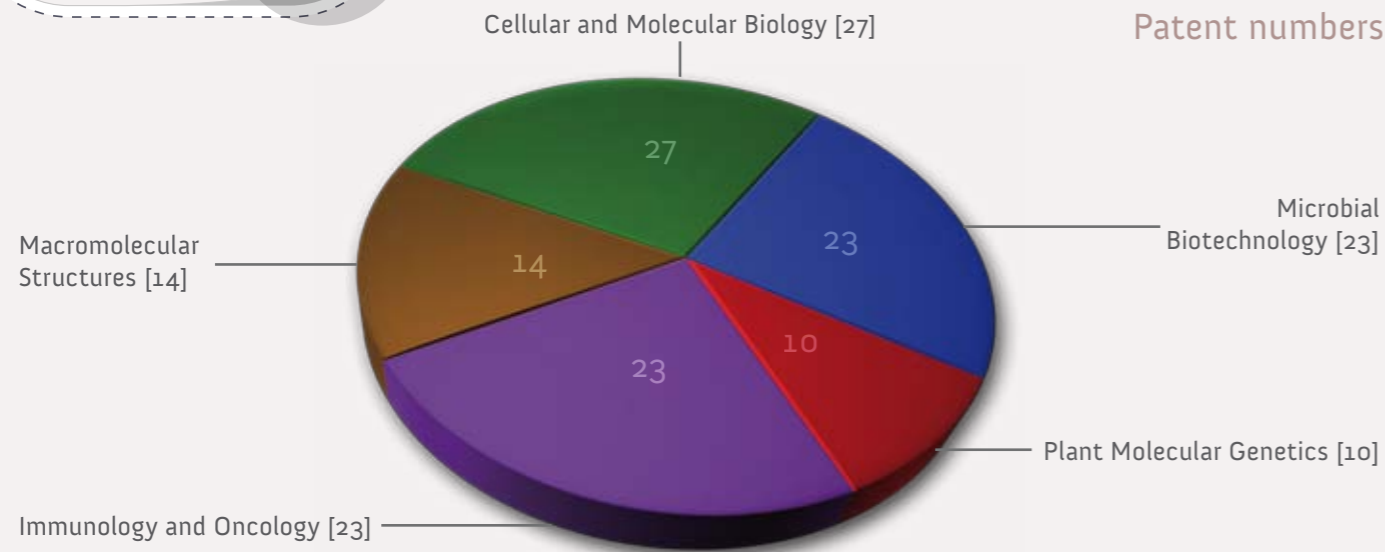


Inder Verma
Proto-oncogenes, gene therapy
Laboratory of Genetics - The Salk Institute for Biological Studies, La Jolla, CA, USA.



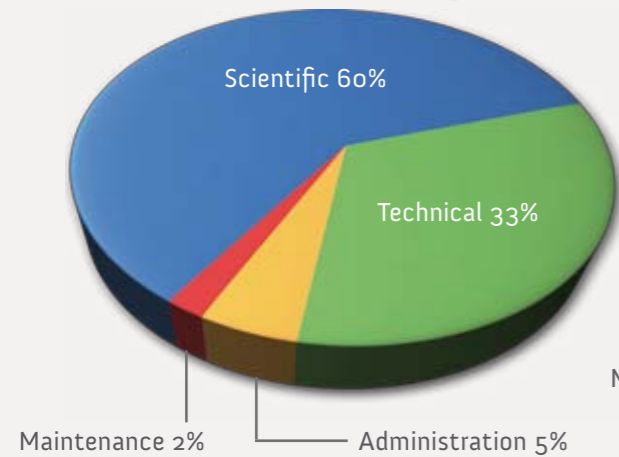
CNB Patent Portfolio

With about 10% of all CSIC patents, we currently have 95 active patent families, of which 70% are international. CNB patents are distributed equally among our research areas:

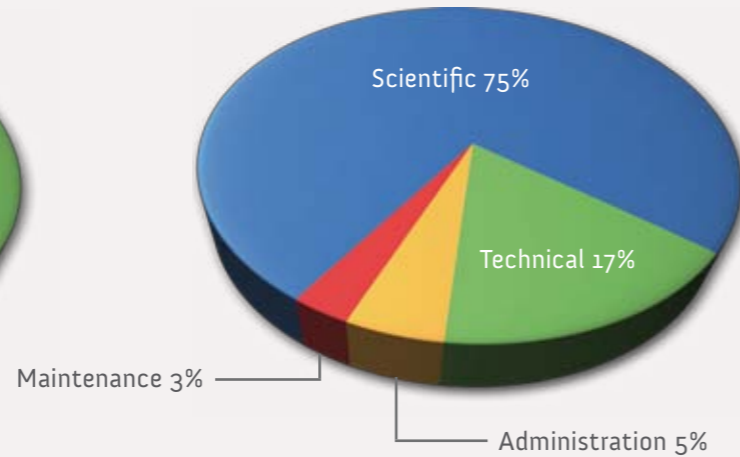


CNB Staff 2009-2010

2009



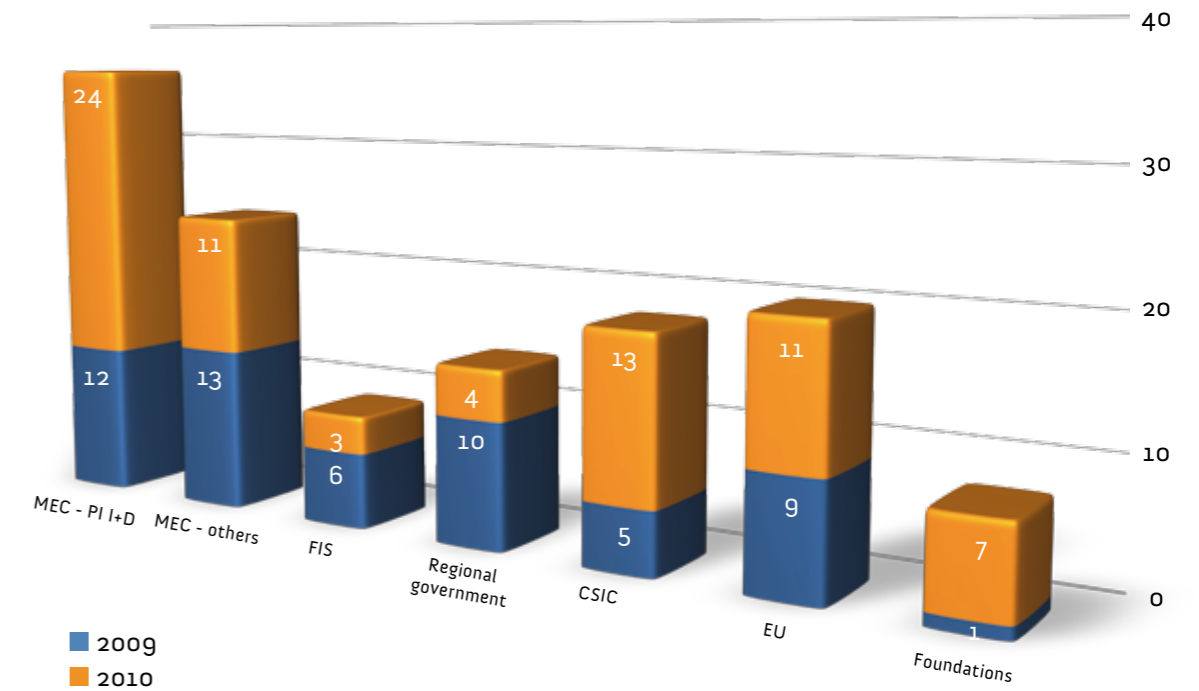
2010



2009 Total Staff	601	2010 Total Staff	597
Scientific	60%	Scientific	75%
Technical	33%	Technical	17%
Administration	5%	Administration	5%
Maintenance	2%	Maintenance	3%
Foreign staff	128 (21%)	Foreign staff	131 (22%)

Research Projects - Total Number

The projects granted to CNB researchers by the European Union (EU) represent 12% of all the projects granted by this institution to the CSIC.



2009

Projects granted

Nº. Amount (€)

MEC - PI I+D	12	2,307,400.00
MEC - others	13	1,800,850.00
FIS	6	613,760.49
Regional government	10	408,856.00
CSIC	5	11,000.00
EU	9	5,019,467.80
Foundations	1	20,000.00

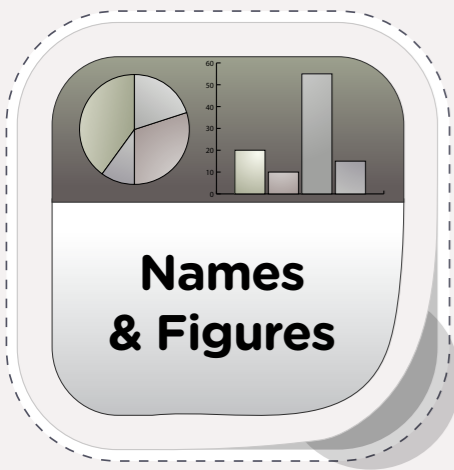
2010

Projects granted

Nº. Amount (€)

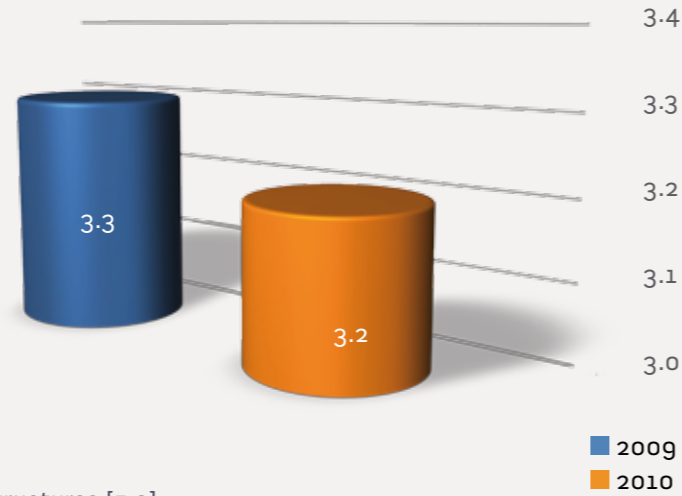
MEC - PI I+D	24	5,678,100.00
MEC - others	11	1,596,455.20
FIS	3	764,850.00
Regional government	4	707,333.40
CSIC	13	212,300.00
EU	11	3,976,336.00
Foundations	7	700,103.00

TOTAL 56 10,181,334.29 73 13,635,477.60

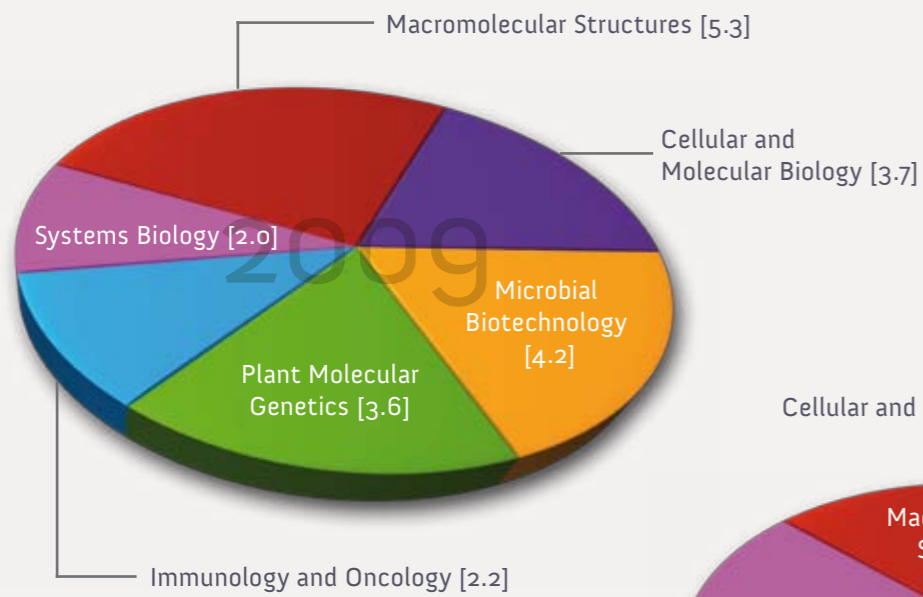


Scientific Publications

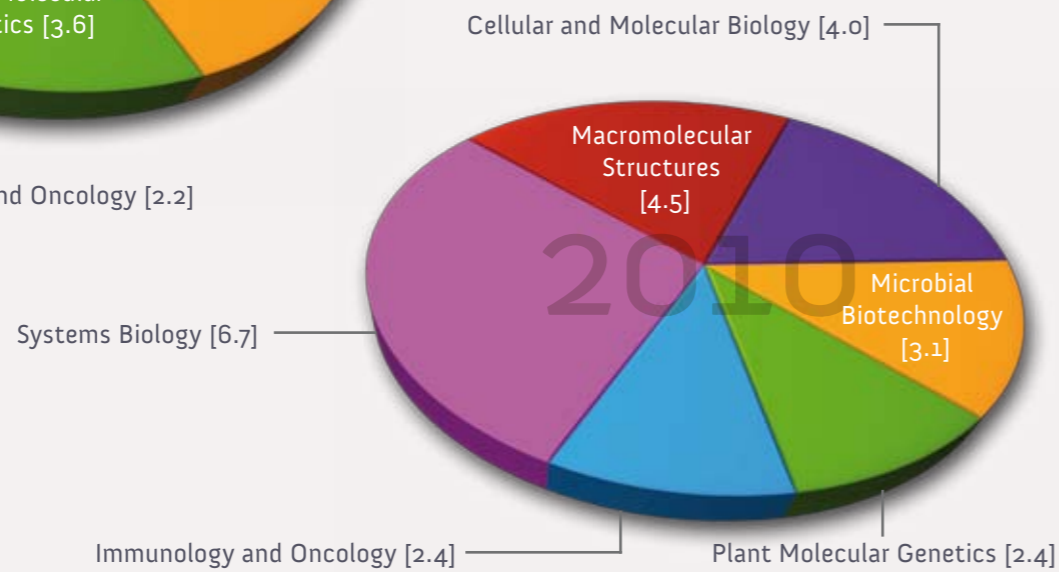
Articles per group



Articles per group 2009



Articles per group 2010



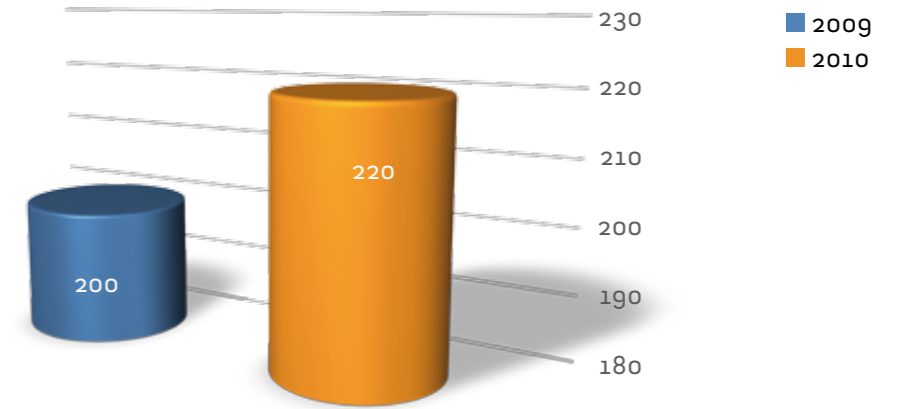
2009 Articles per group

Macromolecular Structures	5.3
Cellular and Molecular Biology	3.7
Microbial Biotechnology	4.2
Plant Molecular Genetics	3.6
Immunology and Oncology	2.2
Systems Biology	2.0

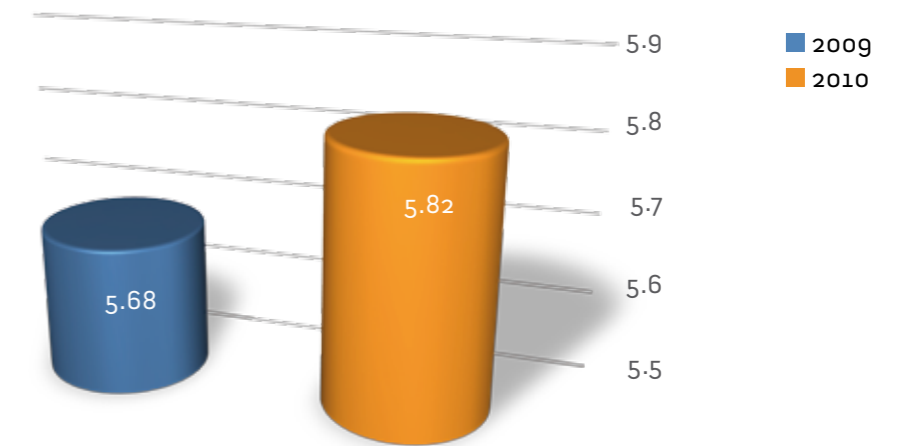
2010 Articles per group

Macromolecular Structures	4.5
Cellular and Molecular Biology	4.0
Microbial Biotechnology	3.1
Plant Molecular Genetics	2.4
Immunology and Oncology	2.4
Systems Biology	6.7

Number of publications



Average impact factor



Highlighted Journals

- | | |
|-------------------------------------|--|
| 1 Science | 4 Nature Structure & Molecular Biology |
| 2 Nature | 1 Neuron |
| 1 Cell | 1 Genes & Development |
| 1 Nature Biotechnology | 1 TIBS |
| 1 Nature Immunology | 2 Blood |
| 1 Nature Cell Biology | 1 Immunological Reviews |
| 1 Journal of Clinical Investigation | 1 Structure |
| 2 Journal of Experimental Medicine | 4 PLoS Genetics |
| 1 Nature Chemical Biology | 5 Journal of Virology |
| 1 Annual Review of Microbiology | 14 Proceedings of the National Academy of Sciences USA |

Scientific Report CNB 2009-2010

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